University of Alberta

Residual Feed Intake in Beef Cattle: Relationships with expression of Duodenal and Jejunal GLUT2, SGLT1, PEPT1, and Ghrelin

by

Steven Guercio

A thesis

submitted to the faculty of graduate studies and research in partial fulfillment of the requirements for the degree of Master of Science

in

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Abstract

Intestinal glucose transporters GLUT2 and SGLT1, peptide transporter PEPT1 and the appetite stimulating hormone Ghrelin were examined to determine if differences in their expression exist between steers with differing residual feed intakes (RFIs). Duodenal and jejunal tissues from the steers were used to examine the differences in the expression of these genes. The level of expression of the genes of interest was measured using the realtime polymerase chain reaction (PCR), and Ussing chambers were used in order to measure actual glucose transport within the duodenal and jejunal samples. The realtime PCR results determined that there was no significant difference between steers with high and low RFIs with regard to gene expression of GLUT2, SGLT1, PEPT1, and Ghrelin (in both duodenum and jejunum). The results from the Ussing chamber experiments also showed no significant differences in the actual transport of glucose between the different steers.

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List of Abbreviations

ADG	average daily gain
AgRP	agouti related protein
C	carbon
Ca	calcium
CCK	cholesystekinin
cDNA	complementary dioxyribonucleic acid
Cl	chlorine
CNS	central nervous system
Co	cobalt
CO_2	carbon dioxide
Cr	chromium
Ct	threshold cycle
Cu	copper
DE	digestible energy
DMI	dry matter intake
ΔRn	magnitude of the fluorescent signal
EFI	expected feed intake
EID	electronic identification
F	fluorine
FAM	fluorophore label
Fe	iron
FRET	fluorescence resonance energy transfer
G	tissue ion conductance
GAPDH	glyceraldehyde phosphate dehydrogenase
GE	gross energy
GH	growth hormone
GHR	growth hormone receptor
GHRF	growth-hormone releasing factor
GHS	growth-hormone secretagogue
GHS-R	growth hormone-secretagogue receptors
GI	gastrointestinal
GIT	gastrointestinal tract
GLUT	facilitated glucose transporter
H	hydrogen
H_20	water
HIF	heat increment of feeding
I	iodine
ICV	intercerebroventricular
IGFBP-4	insulin like growth factor binding protein-4

IgG	immunoglobulin
I_{sc}	short-circuiting' current
kg	kilogram
LGIT	lower gastrointestinal tract
LSMEANS	least squares means
Lys	lysine
ME	metabolizable energy
MEI	metabolizable energy intake
Mg	magnesium
MGB	minor groove binding
Mj	megajoules
MMIDWT	metabolic midweight
Mn	manganese
Mo	molybdenum
mRNA	messenger ribonucleic acid
µl	microlitre
mV	millivolts
Na	sodium
NaCl	sodium chloride
NEg	net energy for gain
NEm	net energy for maintenance
NFE	net feed efficiency
ng	nanogram
Ni	nickel
NPY	neuropeptide Y
NRC	national research council
O	oxygen
OD	optical density
OFI	observed feed intake
oPEPT1	ovine peptide transporter 1
P	phosphorus
PAPP-P	pregnancy-associated plasma protein-A
PCR	polymerase chain reaction
PD	transepithelial potential difference
PEPT	peptide transporter
PKCβII	protein kinase C beta 2
RE	retained energy
RFI	residual feed intake
RIA	Radio immunoassay
RNA	ribonucleic acid

RT	reverse transcriptase
S	sulpher
SAS	Statistical Analysis System
Se	selenium
SGLT	sodium coupled glucose cotransporter
Si	silicon
SRIF	somatostatin
T_m	annealing temperature
UGIT	upper gastrointestinal tract
VFA	volatile fatty acid
VIC	fluorophore label
Vm	membrane potential difference
Zn	zinc
ΔCt	level of expression

1. Literature Review

1.1 General Introduction

The utilization of nutrients by animals in the same species can differ between individuals. Thus, individual cows can vary in terms of utilization of nutrients provided by feed (Archer *et al.*, 1998; Arthur *et al.*, 1998; Okine *et al.*, 2001). The most expensive input in the livestock industry is feed (Fan *et al.*, 1995), about 70% of the total cost of a finishing operation (Liu *et al.*, 2000). About 89% of that 70% of the total cost goes to the energy input of the feed, for example grains (Liu *et al.*, 2000).

A large portion (60-75%) of the total dietary requirements consumed by cattle goes to body maintenance (Ferrell and Jenkins, 1984; Okine *et al.*, 2003). Variations in maintenance and utilization of feed do exist between different animals (Luiting *et al.*, 1994). It is important that distinctions be made between those animals that utilize the feed differently.

Net feed efficiency (NFE) is the measure of how well animals utilize nutrients for maintenance. This is different from feed efficiency, which examines the affect of intake on that of gain (feed to gain ratio). An animal has a high net efficiency when it has a high production level and low body maintenance level (Luiting *et al.*, 1994). This can be converted into an actual number in kilograms referred to as residual feed intake (RFI), which is independent of growth and maturity patterns (Okine *et al.*, 2001). Residual feed intake estimates the efficiency of use of feed consumed by subtracting observed dry matter intake (DMI) of an individual from DMI predicted by an equation developed from the relationship between DMI, daily gain and metabolic mean weight across fed groups

(Basarab *et al.*, 2003; Okine *et al.*, 2003). An animal with a negative RFI value would be considered efficient, while one with a positive value would be inefficient.

There are many factors that affect dry matter intake, one of which is an acylated peptide located in the small intestine, called ghrelin (Hayashida *et al.*, 2001). This peptide is associated with releasing growth-hormone (GH) as well as controlling intake (Kojima *et al.*, 1999; Date *et al.*, 2000; Hayashida *et al.*, 2001; Ariyasu *et al.*, 2002). If it could be shown that there is an association between steers with negative RFI and levels of ghrelin located in the small intestine, this peptide could help to explain the variability between animals with different RFI values.

There are also physiological factors that affect RFI, e.g. nutrient absorption and transport between membranes. The small intestine is the major site for nutrient absorption in cattle (Ruckebusch *et al.*, 1991). Nutrient transporters located in the small intestine that are responsible for major absorption of glucose (GLUT2 and SGLT1), as well as those responsible for the uptake of di and tri-peptides (PEPT1), are important components to examine in order to be able to identify the necessary traits that affect feed utilization.

The purpose of this thesis is to determine if there is a relationship between glucose transporters (GLUT2 and SGLT1), a peptide transporter (PEPT1), and ghrelin, with residual feed intake, by using gene expression techniques and transport measurement, in conjunction with feeding techniques.

1.2 Feed Measurement Trends

1.2.1 Average Daily Gain

Average daily gain (ADG) refers to the average amount of weight that an animal increases by every day that it grows. It is a measurement easily taken by weighing the animal before it is put on a particular diet or as soon as it's born and weighing it again at the end of the feeding period. This method of measuring the average daily gain is highly inaccurate. A more precise method of measuring ADG would be weighing the animals at regular intervals, for example, every week, and regress these values over the entire feeding period. This would account for variations that could occur over the time period such as, the animal getting sick and not eating.

1.2.1.1 Factors that Affect ADG

Sharma *et al.* (1982) found that several factors affect ADG. The objectives of this particular study were to determine the effects of sex of calf and age of dam on birthweight, preweaning average daily gain and 180 day weaning weight, and to determine the effects of age of dam upon postweaning average daily gain and yearling weight in males and 19 month weight in females. The last objective for this experiment was to derive adjustment factors and appropriate methods of applying these factors to the data. The data that was used for this experiment was based on data collected between 1965 and 1978 on two herds of beef cattle. The results showed that there were significant ($P<0.01$) differences for factors such as breed, sex of calf, age of dam and year in birth weight, preweaning ADG and weaning weight. There was a significant ($P<0.05$)

difference between the postweaning ADG and yearling weight in males and 18-month weight in females.

The problem associated with average daily gain, as demonstrated by Sharma *et al.* (1982), is that there are so many factors that contribute to it, that it becomes extremely variable. Although average daily gain reflects how much the animal grows it does not show how much an animal eats or the composition of the gain. An animal could have an impressive average daily gain, but at the same time the reason for this may be that it eats more than the other animals. Another problem with ADG is that it does not indicate what the animal has done with all of the feed it has eaten i.e. how the energy from the feed was utilized, or stored. The animal could have put it towards muscle growth and thus be ideal for sale to a slaughter facility, however the animal could have put the feed into fat, thus affecting price, and decreasing profit. Information provided by the National Research Council (1984, and 2000) indicates that the net energy required for gain increases as ADG increases and as the weight of the animal increases, the same is true for the percentage of fat in gain, and that of body fat; however the opposite is true when considering the percentage of protein in gain. ADG is presently being used combined with other measurements such as feed efficiency to determine the potential profitability of different animals.

1.2.2 Feed Efficiency

Feed efficiency has been studied for many years and it and its inverse, the feed conversion ratio, are widely discussed in the literature (Archer *et al.*, 1998). Feed efficiency can vary throughout a herd and there is a lack of understanding of the mechanism that is responsible for this variation (Archer *et al.*, 1999). Also known as gross efficiency, feed efficiency is both phenotypically and genetically correlated with aspects of production (Archer *et al.*, 1999). This means, for example, certain breeds of cattle that grow faster, are correlated with efficiency of feed intake.

Feed efficiency is the ratio of average daily gain to dry matter intake (Okine *et al.*, 2001). Feed efficiency is an important measure of the ability of an animal to grow in terms of the amount of feed it is given. If an animal has a high feed efficiency then it uses more feed for growth and disposes of less. The opposite is true for an animal with poor feed efficiency. Okine *et al.* (2003) demonstrated that a 5% increase in feed efficiency has about up to 7-8 times the economic impact as a similar increase in the weight of cattle, thus demonstrating that concentrating on feed efficiency in terms of cattle production is necessary if costs are to be decreased. Problems with feed efficiency exist because it is highly positively correlated with growth rate, and could be confounded with growth rate and maturity patterns, body size, composition of gain and appetite (Mrode *et al.*, 1990; Fan *et al.*, 1995; Liu *et al.*, 1998; Arthur *et al.*, 2001; as cited by Okine *et al.*, 2003). This means that feed efficiency is affected by all of these traits, and thus is highly variable. Feed efficiency also demonstrates how an animal can make the most of the amount of nutrients provided, but it does not show if the animal actually needs all of those nutrients (Okine *et al.*, 2001). Two methods used to measure the amount of feed an animal requires for maintenance and growth, are net feed efficiency (NFE) and residual feed intake (RFI). It is important to note that these measurements are independent of growth and maturity patterns, which would enable a more accurate measurement of efficiency. These measurements may be used to identify animals requiring less feed which in turn might be used for breeding populations of more efficient cattle. This, in the long run, will decrease feed costs, because these cattle will utilize their feed more efficiently, thus requiring less.

1.2.3 Net Feed Efficiency and Residual Feed Intake

In the cattle production industry, animals are selected for high production traits (size, growth), which in turn causes an increase in feed costs (Luiting *et al.*, 1994). Feed efficiency depends on the traits of not only the slaughter herd, but the breeding herd as well (Archer *et al.*, 1999). Shuey *et al.* (1993) suggested that there would not be an improvement in the weaned calf weight/unit energy consumed unless the nutrients and feed supplied to the heifer, or cow was limited. This means feeding the breeding generation less in order to achieve a slaughter generation that can consume less feed and still grow to a healthy slaughter weight. They still need to be provided with the amount of feed required to sustain a healthy pregnancy, or if they were not pregnant, to sustain adequate body maintenance.

Net feed efficiency is a method that can be used in order to determine how much feed an animal would require in order to sustain itself. Net feed efficiency is calculated by taking the animal's average daily gain divided by its daily metabolizable energy intake for gain (Okine *et al.*, 2001). An animal's daily metabolizable energy intake for gain is the amount of energy from feed that it uses to grow.

Residual feed intake (RFI) is a method that compares the observed efficiency of utilization of metabolizable energy (ME) and the predicted efficiency of ME utilization (Okine *et al.*, 2001). Okine *et al.* (2001) analyzed the efficiency of utilization of feeds on the basis of gross and net feed efficiency and RFI in steers (60) fed alfalfa, or fenugreek silage with different metabolic energy concentrations, but similar crude protein values and when fed three levels of barley grain to provide different energy densities. The RFI, NFE, gross feed efficiency, metabolic energy intake, metabolic energy required for maintenance and liveweight gain, dry matter intake, average daily gain, and feed to gain ratio were all measured for the different diets. They hypothesized that RFI can be used to determine that differences in metabolizable energy intake by an animal may not necessarily translate into differences in feed efficiency. This was due to the fact that there were no differences in feed to gain ratios between the alfalfa and fenugreek even though there were differences in metabolizable energy intakes, which may be explained by the positive and negative RFI of the cattle on the alfalfa and fenugreek diets.

RFI is phenotypically independent of the production traits used to calculate expected feed intake (Arthur *et al.*, 1998). This means it is independent of factors such as average daily gain and mid weight (Archer *et al.*, 1998). A positive RFI means energy intake exceeds what the animal is predicted to be required to eat, while a negative RFI means that the animal either requires less energy or it can eat less to achieve the same weight (Okine *et al.*, 2001).

Phenotypic and genetic variation exists in the feeding efficiency of growing cattle (Arthur *et al.* 1998). This evidence also adds to the fact that there are phenotypic and genetic differences in the residual feed intake of growing cattle (Arthur *et al.*, 1998). Because these traits are genetic, this means that they can be passed on from generation to generation. In terms of adult cattle, efficiency has two sides: efficiency at maintenance and efficiency during lactation (Arthur *et al.*1998). A cow requires more feed when it is going through lactation because of the requirements for body maintenance and the requirement of energy for milk production. There is a genetic variation in efficiency among lactating dairy cows (Arthur *et al.*, 1998). If one cow can produce the same amount of milk as another, but requires less feed, it would be considered to have a negative residual feed intake. This same principle can be applied to beef cattle. If a calf can grow at the same rate as others, while its dam eats less, then it is beneficial for that cow to be bred further. The calf should have some of the same traits as the dam, therefore it could be able to eat less and still grow at the same rate as the other cattle.

In a study performed by Archer *et al.* (1998), a total of 1166 cattle of 116 sires were tested from 1994 to 1997 to predict responses in component traits to selection using residual feed intake as an index of efficiency. Each test period lasted 119 days. It was determined that RFI is not phenotypically correlated with ADG and mid-weight, while the genetic variation in growth performance was negatively correlated with ADG, and was positively correlated with feed intake. This means that if the animals were selected for RFI, one would decrease feed intake, thus improving efficiency and feed conversion. The implication of this selection method would be to decrease the costs required to feed the cattle.

Richardson *et al.* (2001) studied differences in feed intake, growth, body composition and implications for heat production of Angus steer progeny from parents that were selected for and against post-weaning residual feed intake (RFI). It was determined in this experiment that low RFI steers gained more protein than the high RFI steers. The low RFI steers were also accompanied with a greater proportion of protein in the final liveweight than the high RFI steers. Richardson also determined that there was a positive correlation between RFI and end-of-test chemical fat and a negative correlation with chemical protein. This implies that steers with a low RFI will have a lower chemical fat and a higher chemical protein at slaughter. There was also a significant difference ($P<0.05$) in carcass fat between the high and low RFI animals, the low RFI steers having less carcass fat than the high. The relationship between RFI and carcass fat carries a significant ($P<0.01$) correlation of 0.47. There was no significant difference between high and low RFI animals when the percentage of retail beef was considered, however the correlation of –0.36 between RFI and percent retail beef was considered to be significant ($P<0.05$). The difference in RFI between the high and low RFI steers was 0.31 kg DM/day, 3.8% of the average daily DMI for all of the steers.

A study by Basarab *et al.* (2003) looked at the relationship between RFI and body composition. They found a positive relationship between RFI and gain in empty body fat ($r=0.26$, $P<0.01$), implying that animals with low (or negative) RFI values deposit empty body fat at a slower rate. Other components of this study determined that there were positive relationships found between RFI and carcass marbling ($r=0.14$, $P<0.08$), dissectible carcass fat ($r=0.14$, $P<0.10$), gain in ultrasound backfat thickness ($r=0.22$, $P<0.01$), gain in ultrasound marbling ($r=0.22$, $P<0.01$), gain in empty body fat ($r=0.26$, $P<0.01$), metabolizable energy intake (MEI) ($r=0.80$, $p<0.01$), retained energy (RE) ($r=0.28$, $P<0.01$), and heat production ($r=0.56$, $P<0.01$). Negative relationships were found between RFI and dissectible carcass lean ($r=-0.21$, $P=0.01$), and empty body protein ($r=-0.14$, $P<0.10$). This study shows that not only does RFI impact feed intake traits, but body composition traits as well, however, the relationships found in this study are small, in terms of the low r values. Thus, although an animal may have a high RFI, the effect that this will have on its carcass marbling is minimal.

Residual feed intake is calculated by subtracting an animal's expected feed intake from its observed feed intake (Basarab *et al.*, 2002). In order to determine observed feed intake, some initial values are needed. Total intake is calculated by multiplying the average intake by the number of days on the trial. This value is then converted to a dry matter basis (DMI), and then to total metabolizable energy intake (MEI). This is done by multiplying the total DMI by the metabolizable energy of the diet. The MEI is then standardized by adjusting each animal's MEI into standard feed intake at 10 MJ/kg. This is to determine how much the animal would have eaten if the ME of the diet were 10 MJ/kg, and is expressed in kilograms. The total standard feed intake is then converted into daily standard feed intake by dividing by the days on trial, this is also known as observed feed intake (OFI).

In order to calculate expected feed intake (EFI) the average daily gain (ADG) and the metabolic midweight (MMIDWT) for each animal must be calculated. This is done by regressing the weight of each animal on time (days). The negative days (days before trial) are also included to smooth out rates of weight gain. The intercept of the regression curve is the day zero weight, and the slope of the regression curve is average daily gain. Metabolic mid weight is calculated by the following formula:

$$\text{MMIDWT} = (\text{day 0 weight} + (\text{days}/2*\text{ADG}))^{0.75}$$

After ADG and MMIDWT are calculated they are regressed on observed feed intake, i.e.:

$$\text{Observed feed intake} = \text{ADG} + \text{MMIDWT}$$

This is done to obtain an intercept for the whole model (a_0), the slope of observed feed intake on ADG (b_1), and the slope of observed feed intake on MMIDWT (b_2). Following which the expected feed intake was calculated for each individual animal as:

$$EFI = a_0 + (b_1{*}ADG) + (b_2{*}MMIDWT)$$

After OFI and EFI have been calculated the RFI for each animal can be determined by the following formula:

$$RFI = OFI-EFI$$

Animals that have a higher expected feed intake than the observed are considered to be efficient (i.e. need less feed for body maintenance and production), while the opposite is true for animals with lower expected feed intake than the observed.

In summary, residual feed intake is a way of determining if the animal needs to be fed more, less, or the same. This can be used as a selection trait as shown by Richardson *et al.* (2001). If the RFI is known for a bull or a cow, then that can be used to predict the RFI of the offspring. This technique, if used on a larger scale, could help in the reduction of feed costs.

1.3 Factors that Affect Residual Feed Intake

Some examples of contributing factors to differences in RFI are aspects related to dry matter intake (DMI) such as the different hormones, for example cholesystekinin (CCK), insulin, leptin, and, neuropeptide Y (NPY), as well as recently discovered acylated peptide located in the small intestine (ghrelin). All of these hormones effect intake (Stanely *et al.*, 1986; Woods *et al.*, 1998; Forbes, 2000; Hayashida *et al.*, 2001; Liefers *et al.*, 2003), and could have an influence on the RFI of an animal. Ghrelin is associated with releasing growth-hormone (GH), and appears to have influential affects on feed intake (Kojima *et al.*, 1999; Date *et al.*, 2000; Hayashida *et al.*, 2001; Ariyasu *et al.*, 2002). Studies show that there are high levels of plasma ghrelin in the blood before a meal and during fasting, and decreases following feed intake, as well as high levels were detected in obese animals (Hayashida *et al.*, 2001; Ariyasu *et al.*, 2002).

The ability to identify how an animal will utilize feed would be considered an asset to the selection process. There are many different physiological factors involved in feed utilization that could contribute to differences in RFI between animals. Aspects such as digestible energy, protein and lipid synthesis, size of internal organs, and nutrient transport (Ferrell and Jenkins, 1998; Kühn *et al.*, 2002; Ferrell, 2003; Kellems and Church, 2002; NRC, 2000). Nutrient transporters are responsible for mediating the transport of nutrients between membranes (i.e. absorption) (Doring *et al.*, 1997), and the small intestine is the major site for nutrient absorption in cattle (Ruckebusch *et al.*, 1991). The intestinal glucose transporters GLUT2 and SGLT1, as well as the intestinal peptide transporter PEPT1 have the potential to assist in the explanation of the variability of RFI in terms of nutrient absorption and transport.

1.3.1 Hormonal Factors that Affect Dry Matter Intake

There are a number of hormones that influence dry matter intake in cattle. These hormones range from those located within the gastrointestinal tract to ones located in the central nervous system (i.e. brain). This section will discuss briefly the major hormones that affect DMI (i.e. neuropeptide Y, cholesystokinin, insulin, and leptin).

Neuropeptide Y (NPY) is a 36-amino acid peptide located throughout the CNS (central nervous system), but is mainly concentrated within the hypothalamus (Nandi *et al.*, 2002). The NPY containing cell bodies within the hypothalamic arcuate nucleus are influential in the regulation of feed intake. It has been shown that NPY administered into the hypothalamus on a regular basis leads to obesity (Stanley *et al.*, 1986). The release of NPY can be inhibited and partially regulated by the release of leptin from adipose tissue, as well as the release of insulin (Nandi *et al.*, 2002). Other factors also affect the release of NPY, one of them being the recently discovered hormone, ghrelin (to be discussed in the next section). The external modification of the production of NPY would not be feasible when dealing with large animals such as cattle, especially when dealing with large herds of them. Other methods of controlling feed intake must be looked at when considering production animals.

Cholecystokinin (CCK) is 33-amino acid containing peptide, released in response to the end products of fat and protein digestion (Porte and Halter, 1985). It is produced in the Islet cells of the duodenal and jejunal mucosa, and is responsible for provoking bile secretion and zymogen (inactive form of an enzyme activated by acid) release from the acinar cells of the pancreas. Cholecystokinin stimulates abdominal receptors which transmit information to the CNS via the vagal pathway (Forbes, 2000). It has been shown to depress intake when administered intraperitoneally. However it is not feasible to administer CCK intraperitoneally to livestock, as well as the fact that it has been shown to decrease intake, and makes no mention of energy partitioning. The animal might eat less, but this animal might be one that is not over eating and administering

CCK might hinder its growth and development. The animal, because of the administration of CCK, may not eat what is necessary for it to maintain growth.

Insulin is produced within the small percentage (5%) of the pancreas referred to as Islet Cells. Insulin is composed of two peptide chains (A and B). Chain A consists of 21-amino acids, while chain B consists of 30-amino acids. The two chains are linked by two disulfide bridges between positions 7 and 7 of the A and B chains respectively and between positions 20 and 19 of the A and B chains respectively (Porte and Halter, 1985). Insulin is produced by the beta cells while the alpha cells produce its antagonist glucagon. Insulin is secreted in response to high sugar levels in the blood and food consumption, and is regulates fat deposition within adipose tissue. Its main function is the storage of nutrients, and has an intricate role in the synthesis of fats, formation of liver and muscle glycogen, and protein synthesis (Davis *et al.*, 1998). The depletion of energy stored in the form of adipose tissue, will cause an increase in food consumption. In a state of negative energy balance, adipose tissue mass will contract, causing less leptin and insulin to be transmitted to the brain thus increasing intake (Woods *et al.*, 1998). When animals are treated with insulin in the hopes of decreasing intake, the opposite occurs causing an increase in intake, thus implying that insulin alone is not solely responsible for controlling intake (Forbes, 2000).

Leptin is a 146-amino acid peptide that is also secreted by adipose cells, and as stated previously influences feeding behaviour. Liefers *et al.* (2003) found that plasma leptin concentrations were lower in dairy cows with a mean negative energy balance during lactation and that those cows had a higher milk yield, and had a lower intake and live weight when compared with cows having a mean positive energy balance. This implies that animals with low plasma leptin concentrations will have a lower feed intake. This was demonstrated by Amstalden *et al.* (2000) who showed that heifers with restricted feed intake had lower levels of plasma leptin than the control group as well as a lower expression of leptin mRNA within adipose tissue. Both leptin and insulin inhibit brain anabolic neural circuits that stimulate intake and repress energy expenditure (Nandi *et al.*, 2002). Leptin has major influences on feeding behaviour and appears to be an antagonist of ghrelin.

1.3.1.1 Ghrelin

Ghrelin is a growth hormone releasing peptide, consisting of 28-amino acids, recently discovered by a Japanese research group from a rat stomach extract (Kojima *et al.*, 1999). The term ghrelin was chosen based on *ghre* is the Proto-Indo-European root of the word 'grow' and 'relin' refers to "releasing substances". Ghrelin is an endogenous ligand for growth hormone-secretagogue receptors (GHS-R), which are G-protein-coupled receptors. These receptors in turn stimulate the release of growth hormone (GH) from the pituitary gland through small synthetic molecules called growth-hormone secretagogues (GHS).

It was originally assumed that there were two hypothalamic peptides that controlled the release of GH by the pituitary: growth-hormone releasing factor (GHRF) and somatostatin (SRIF) which inhibited the release of GH, however it was always hypothesized that there was another regulatory factor involved (Monamy *et al.*, 1981; Bowers *et al.*, 1984; Bowers ~~*et al.*,~~ 2001; Wang *et al.*, 2002). "It was not until the late 1970s and early 1980s that a series of synthetic factors (i.e. GHSs) were made based upon the structure of a small hypothalamic peptide, metenkephalin, which stimulated GH secretion via the pituitary and hypothalamus" (Wang *et al.*, 2002).

GHSs and GHRH stimulate different populations of pituitary cells (Goth *et al.*, 1992). "If a human is deficient in GHRH this seems decrease the effectiveness of GHSs, implying that exposure of pituitary somatotrophs to GHRH is necessary for GHSs to be maximally effective" (Wang *et al.*, 2002; Pandya *et al.*, 1998). It is also important to note that GHSs require a functional hypothalamus in order to stimulate the release of GH (Popovic *et al.*, 1995).

The majority of studies involving ghrelin have involved rodents and humans, there are very few studies involving other animals. The ghrelin mRNA/peptide has been found in many tissues. The mucosal layer of the stomach is the primary location for ghrelin expression, followed by the antrum, duodenum, ileum, and colon (Wang *et al.*, 2002; Lee *et al.*, 2002; Hosada *et al.*, 2000b; Dornonville de la Cour *et al.*, 2001; Date *et al.*, 2000). Ghrelin is also produced in the pancreas, kidney, liver, hypothalamus, pituitary, and in immune cells (Wang *et al.*, 2002; Kojima *et al.*, 1999; Korbonits *et al.*, 2001; Mori *et al.*, 2000; Hattori *et al.*, 2001). Gualillo *et al.* (2001) found the mRNA/peptide to be expressed in rat and human placenta. The receptor for ghrelin (GHS-R) has been found in the pituitary, hypothalamus, stomach, heart, blood vessels, lung, pancreas, intestine, kidney, adipose tissue, immune system and in human breast carcinomas (Wang *et al.*, 2002; Nagaya *et al.*, 2001; Hattori *et al.*, 2001; Guan *et al.*, 1997; Papotti *et al.*, 2000; Shuto *et al.*, 2001; Cassoni *et al.*, 2001). In terms of the receptor expression in the brain, it was highest in the hypothalamus, hippocampus formation, and the pituitary.

Ghrelin has two major molecular forms: ghrelin and des-Gln14-ghrelin (Hosoda *et al.*, 2000a). The two forms are identical except des-Gln14-ghrelin has a deletion of Gln14, resulting in a CAG codon, which encodes Gln14 as a splicing signal, and also appears to have the effects on the release of growth hormone. Ghrelin, however, is present in larger amounts in the stomach, thus it is the major active form (Hosoda *et al.*, 2000b).

As stated previously ghrelin stimulates the release of GH, however it has also been shown to stimulate appetite (Tschöp *et al.*, 2000; Nakazato *et al.*, 2001; Shintani *et al.*, 2001; Wren *et al.*, 2000; Wren *et al.*, 2001; Hayashida *et al.*, 2001). The main active site of ghrelin is the hypothalamic arcuate nucleus which is also the target site for the hormone leptin (appetite suppressor). An intercerebroventricular (ICV) injection of ghrelin induced the expression of Fos protein (a marker for neuronal activation) in NPY-containing neural cells and caused an increased the level of NPY mRNA in the arcuate nucleus (Nakazato *et al.*, 2001). The appetite stimulating effects of ghrelin are inhibited by NPY receptor (Y_1) antagonist, AgRP inhibitor, anti-NPY IgG, and anti-AgRP-IgG. "These results indicate that ghrelin increases feeding activity by stimulating NPY- and AgRP-containing neurons in the hypothalamus to promote the production and secretion of NPY and AgRP peptides, and is thus a natural antagonist to leptin" (Kojima and Kangawa, 2002).

Levels of ghrelin in plasma are affected by intake. It has been demonstrated that plasma ghrelin levels are high before intake and decrease after intake, are high when the subject is fasting, and are reduced in obese individuals (Hayashida *et al.*, 2001; Toshinai *et al.*, 2001; Ariyasu *et al.*, 2002; Murakami *et al.*, 2002; Sugino *et al.*, 2002). It is important to note that ghrelin levels are not affected by gastric distention with water, implying actual nutrients control the levels of ghrelin (Tschöp *et al.*, 2000). Broglio *et al.* (2002) demonstrated that plasma ghrelin concentrations decreased by feeding meals with high lipid concentration and increased in meals low in response to protein, thus showing that diet can influence ghrelin levels.

One of the aspects of the study done by Hayashida *et al.* (2001) involved measuring the levels of plasma ghrelin before and after feeding seven adult Japanese black cows. The levels of plasma ghrelin were reduced one hour after feeding ($p<0.05$ vs. pre-feeding) and returned to the pre-feeding levels ($p>0.05$ vs. pre-feeding). These results indicate that ghrelin is affected by feed intake of cattle, and thus could be an aid in differentiating between those with high and low residual feed intake. The expression of ghrelin within the GI tract could directly affect the amount of unnecessary feed an animal will consume which could have a relationship with RFI.

1.3.2 Physiological Factors Contributing to Residual Feed Intake

1.3.2.1 Dietary Energy

Dietary energy is broken down into four categories: gross, digestible, metabolizable, and net energy. Gross energy (GE) is the total energy provided by the feed and consumed by the animal. Digestible energy (DE) represents the energy that the animal digests. Digestible energy is the amount of energy remaining, after the energy utilized to produce fecal matter is removed from the gross energy, and this amounts to about 10-60% of the gross energy depending on the digestibility of the feed (NRC, 2000). Factors such as feed quality affect fecal energy. A poor quality feed will increase fecal energy, while a high quality feed will decrease fecal energy (NRC, 2000).

Metabolizable energy (ME) remains after energy is lost through gas production and urination. The formula for metabolizable energy is: ME=DEx0.82 (NRC, 2000). According to the previous calculation energy lost through urine and gas production is about 18% of the digestible energy. Specifically, urinary energy loss accounts for approximately 2-8% of GE and 5-9% of DE (NRC, 2000). Feeds, high in protein content as well as roughages increase urinary losses. Gaseous losses accounts for approximately 6-10% of GE and 8-16% of DE (NRC, 2000). This is caused by poor quality feeds, because gas production (CH_4 and CO_2) decreases proportionally as digestibility increases.

Metabolic energy does not account for heat losses; therefore the final step in the energy system is split into two categories: heat production and retained energy. Retained energy (net energy for gain, NEg) is the energy that the animal puts toward factors for production such as growth and milk production. Heat production is split into two categories; net energy of maintenance (NEm) and heat increment of feeding (HIF). Net energy of maintenance is the energy utilized by cattle for regular body functions such as use of the vital organs. The formula for net energy is NEm=ME-HIF. Heat increment of feed is not easy to measure, but it represents the energy of eating i.e. chewing (approximately 0.3-3.5% of ME), the energy caused by rumination (approximately 2% of ME), the energy caused by digestion (approximately 2.1% of ME), and the energy associated with fermentation of feedstuffs (approximately 7% of ME) (NRC, 2000). Figure 1.3 represents a flow chart of the energy system of a cow.

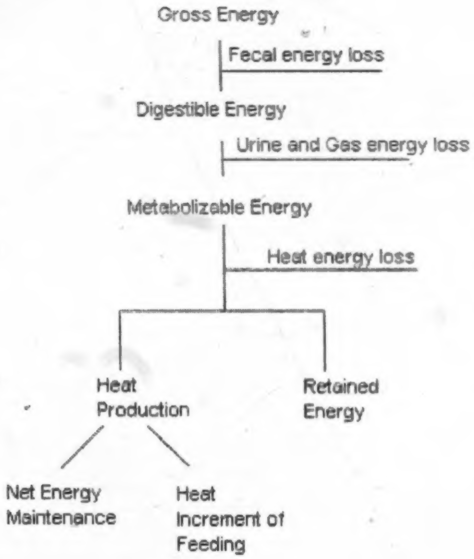

Gross Energy
Fecal energy loss
Digestible Energy
Urine and Gas energy loss
Metabolizable Energy
Heat energy loss
Heat Production
Retained Energy
Net Energy Maintenance
Heat Increment of Feeding

Fig. 1.1. Dietary energy flow chart.

1.3.2.2 Protein and Lipid Synthesis

The accumulation of lipid is more efficient than the accumulation of protein (Ferrell, 2003). Ferrell and Jenkins (1998) found that, animals fed ad libitum had higher protein and fat composition than those limit fed, and although intake increased, rate of gain decreased. The reason rate of gain decreased is because the animals had to put more energy and nutrients into the maintenance of the excess protein and fat, thus in theory affecting RFI. Partial efficiencies of ME use for fat energy gain, are between 60% and 80%, while only between 10% and 40% of ME used for protein energy gain (Ferrell, 2003). Different breeds prioritize the segregation of nutrients and energy for fat and protein synthesis differently (Kühn *et al.*, 2002; Ferrell and Jenkins, 1998). This is a major issue in cattle production, because the majority of herds have crossbred cattle, thus the synthesis effects of each of the contributing breeds will have a prioritizing effect on nutrient segregation. Once it can be established how different breeds distribute energy and nutrients in relation to their metabolizable energy, this will provide incite about nutrient pathways and their regulation (Kühn *et al.*, 2002).

1.3.2.3 Internal Organs

Ferrell and Jenkins (1998) found that the weight of internal organs (stomach complex, intestines, heart, lung, kidney, and spleen) is related to feed intake. Animals that had a restricted feed intake had organs that weighed less than those fed ad libitum (Johnson et al., 1985; Ferrell and Jenkins, 1998). Ferrell and Jenkins (1998) determined that animals with heavier organs have higher metabolizable energy intake. This could partially be due to energy required to maintain the larger organs. The animals would thus be required to eat more to sustain growth. Ferrell and Jenkins (1985) pointed out that "variations in maintenance and efficiency of gain are frequently more highly associated with weight and metabolic activity of visceral organs such as gut and liver rather than body protein and fat". These effects could have a major effect on net feed efficiency because the animals have increase intake, so they can put more energy towards maintaining these visceral organs, thus causing the animal to have a positive residual feed intake value (inefficient).

1.3.2.4 Carbohydrate and Peptide Transport within the Small Intestine

It is important to note that it is more efficient for cattle to retain energy from carbohydrates digested and absorbed directly in the small intestine than retain the energy that is produced by VFAs (Kellems and Church, 2002). This is because the energy retained from the rumen is an end product of microbial fermentation (the carbohydrate must first be fermented by the microbe before being absorbed), while that which is retained from carbohydrates digested in the small intestine is a result of direct absorption of those carbohydrates (Ruckebusch *et al.*, 1991; Kellems and Church 2002). This also depends upon the amount of starch that escapes rumen fermentation. Dietary carbohydrates within the digestive chyme present in the small intestine are absorbed as monosaccharides (Wright, 1993). Mature enterocytes (cells of the intestinal epithelium) line the upper 1/3 of the villi and absorb these monosaccharides, which are in the forms of D-glucose, D-galactose, and D-fructose (Wright, 1993). The first step involves the coupling of sugar transport across the brush border. This is done by 2 glucose transporters: one, which is sodium dependent (SGLT1) and one, which is facilitative (GLUT2) i.e. is controlled by the influx of nutrients to the small intestine and is signaled by SGLT1 (Kellett, 2001). The facilitative glucose transporter then transports the glucose to the basolateral side and into the blood stream (Kellett, 2001; Thorens, 1993; Wright, 1993).

Dietary proteins are broken down into peptides and amino acids by pancreatic proteases (Daniel, 1996). The absorption of di and tripeptides occurs in a similar manner. Proton coupled transporters transport di and tripeptides from the brush border membrane to the basolateral side of the small intestine and into the blood stream (Daniel, 1996).

How an animal transports nutrients could reflect on the utilization these nutrients, thus indirectly affecting intake. Efficiency of transport could be considered as the amount of nutrients that are absorbed compared to the amount of nutrients that are consumed. If it could be determined which animals utilize more efficiently the nutrients provided in the feed (through the different transporters), this could have a direct relationship to residual feed intake.

Differences exist in the expression of the glucose transporters GLUT2 and SGLT1 (Zhoa *et al.*, 1993; Zhao *et al.*, 1998 respectively) as well as the PEPT1 (Chen *et al.*, 1999), throughout the bovine gastrointestinal tract. If differences exist within animals then it is possible that differences could exist between animals. If an animal were to have a high expression of GLUT2, SGLT1, and PEPT1 mRNA within the small intestine, it is conceivable (if the mRNA converted to the transporter proteins) that more glucose and peptides would be absorbed, than that of an animal with low expression of GLUT2 and SGLT1. This could in turn affect RFI by the animal with the higher expression of the transporter genes having higher transport efficiency, thus requiring absorption of fewer nutrients and consuming less feed than the animal with a low expression of these particular genes.

1.3.2.4.1 Glucose Transporters

There are many glucose transporters situated at different locations throughout the body of mammalian species. There are two major types of glucose transporters: SGLT (sodium coupled glucose cotransporter), and GLUT (facilitated glucose transporter). SGLT involves the coupling of glucose by Na+ and the utilization of an electrochemical gradient to transport it across a membrane (Wright, 1993). GLUTs allow passive movement across the plasma membranes down the concentration gradient (Hediger and Rhoads, 1994). The different glucose transporters range from, SGLT1 to SGLT3 and GLUT1 to GLUT11.

SGLT1 has been studied for many years. It has been found to be mainly located in the intestine (Harmon and McLeod, 2001; Hediger and Rhoads, 1994; Wright, 1993; Zhao, 1998), but it is also located in the kidney along with SGLT2 (Vestri *et al.*, 2001). Zhao *et al.* (1998) detected strong expression of SGLT1 in the jejunum and ileum, and weak expression in the duodenum sections of the small intestine of lactating cows. Kong *et al.* (1993) discovered that SGLT3 was expressed in the kidney, intestine, liver, and spleen of pigs.

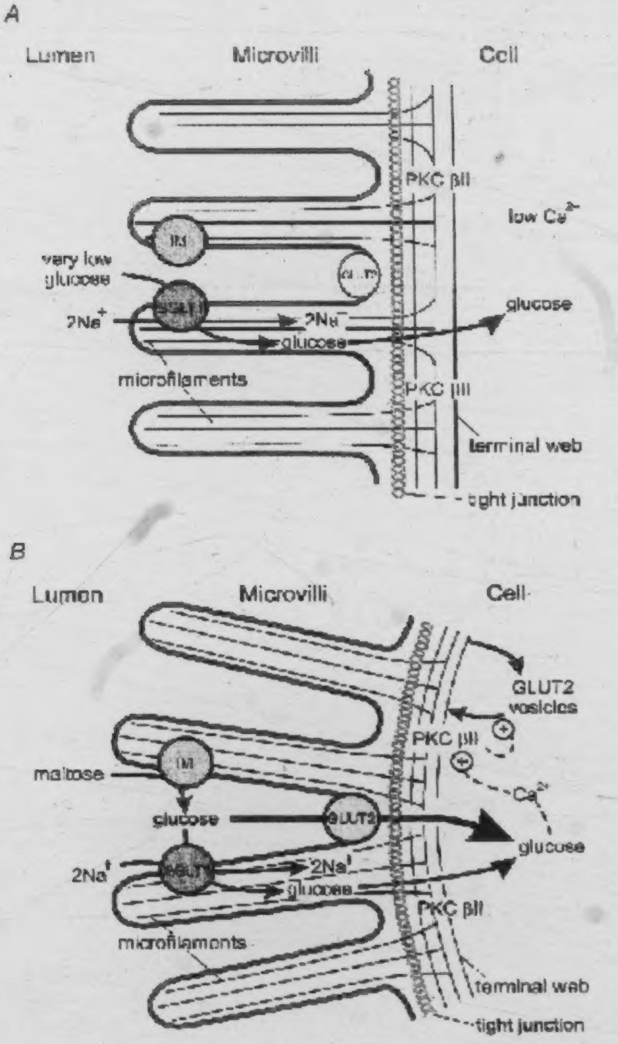
GLUT1 is located in the mammary gland (Zhao *et al.*, 1996) and brain (Simpson *et al.*, 2001) of cattle. GLUT2 is located in the liver, small intestine, kidney, and pancreatic β-cells (Helliwell *et al.*, 2000; Hocquette and Abe, 2000; Kellett and Helliwell, 2000; Olson and Pessin, 1996; Thorens, 1993), while GLUT5 is located solely in the small intestine (Zhao *et al.*, 1998). GLUT3 is located in the human and rodent brain (Maher *et al.*, 1992). GLUT4 was located in the heart, skeletal muscles, and adipose tissue, all of which are insulin responsive tissues (Hocquette *et al.*, 1995). The brain, spleen, and leucocytes of rats contain mRNA for GLUT6 (Lisinski *et al.*, 2001). It is uncertain what the function of GLUT7 is or even if it exists. It was originally thought that GLUT7 encoded a rat liver endoplasmic reticulum glucose transport protein (Mueckler, 1994); this was disproved by Ann Burchell (1998). GLUT8 had the highest expression level in the testis of adult rats, though not pre-pubertal rats (Doege *et al.*, 2000b). GLUT8 was also discovered in lower amounts in muscle, brain, liver, and kidney (Doege *et al.*, 2000b). Doege *et al.* (2000a) detected GLUT9 in the human spleen, peripheral leucocytes, and the brain. GLUT10 was detected in the human heart, lung, brain, liver, skeletal muscle, pancreas, placenta, and kidney by Northern blot analysis

(Dawson *et al.*, 2001). Finally GLUT11 was shown to be transcribed in the heart and skeletal muscle (Doege *et al.*, 2001). It should be noted that these glucose transporters may exist in smaller amounts in other areas of the body. The areas that are mentioned are where they are predominantly expressed.

The glucose transporters to be studied in this thesis are SGLT1 and GLUT2. This thesis deals with glucose transporters in the small intestine, and SGLT1 and GLUT2 are the most likely major glucose transporters in the small intestine of a cow.

SGLT1 and GLUT2 work together to transport glucose across the intestine from the brush border membrane (apical side) to the basolateral side (Zhao *et al.*, 1998). Zhoa *et al.* (1998) examined the expression of SGLT1 in various regions of a lactating cow. Four Holstein dairy cows were used as the tissue donors for this experiment. The use of a Northern blot showed that there was gene expression of SGLT1 in the rumen, omasum, duodenum, jejunum, ileum, and cecum. An immunoblot analysis detected a strong 69-kDa band representing SGLT1 in the jejunum and ileum of lactating dairy cattle, calves, and rats. There were low signals detected in the duodenum.

It was originally observed that SGLT1 was located on the brush border membrane while GLUT2 was located on the basolateral side (Wright, 1993; Thorens, 1993). GLUT2 has, however, been detected at the brush border of diabetic rat jejunum (Corpe *et al.*, 1996). It has also been detected at the brush border of rat jejunum by immmunohistochemistry (Au *et al.*, 2002). When the jejunum is excised for *in vitro* experiments of any kind there is an associated inactivation of PKCβII (Protein Kinase C beta 2) and the rapid loss of GLUT2 from the brush border membrane. This is due to the loss of the influence of stimulating hormones and nutrients (Kellett, 2001). PKCβII is a member of a family of protein serine/threonine kinases activated by phospholipids that play an important part in intracellular signaling (Lackie and Dow, 1997). When a portion of the intestine is excised, it halts the mechanism that controls the signaling of GLUT2 (i.e. PKCβII) to the brush border membrane. It has been shown that PKCβII activity in *in vivo* experiments is controlled by hormones and nutrients (Helliwell *et al.*, 2000). The excision of the intestine prevents these hormones and nutrients from continuing to control PKCβII, thus indirectly controlling GLUT2. GLUT2 can move away from the brush-border within minutes (Helliwell *et al.*, 2000). As a result, it was difficult to detect GLUT2 at the brush border membrane. Trafficking of GLUT2 at the brush border membrane is controlled by the SGLT1 dependent activation of a protein kinase C (PKC) (Kellett, 2001), implying that SGLT1 activates PKCβII. SGLT1 thus indirectly recruits GLUT2 to the brush border membrane. The lumen is empty before a meal and the concentration of glucose is lower than in the blood, as is shown in Figure 1.2a. (Kellett, 2001). The potential loss of glucose from the small intestine at this stage is limited by three factors: the intrinsic activity of GLUT2 at the brush border is low, a large part of glucose metabolized from the blood villi occurs at this point, and finally the glucose that does escape from the membrane is recycled by SGLT1 (Kellett, 2001). After a meal, the concentration of glucose will increase as a result of the action of membrane-bound hydrolytic enzymes (e.g. isomaltase). Isomaltase converts the disaccharide maltose into glucose. There are similar enzymes located on the villi to break down other dissacharides into glucose. The transport of glucose will initially occur through SGLT1 which activates PKCβII which, in turn, causes GLUT2 to respond to the influx of glucose (Kellett, 2001). This series of events are shown in Figure 1.2b. The large arrow indicates


A
Lumen
Microvilli
Cell
PKC βII
low Ca^{2+}
IM
very low glucose
GLUT
SGLT1
2Na$^+$
2Na$^+$
glucose
glucose
microfilaments
PKC βII
terminal web
tight junction
B
Lumon
Microvilli
Cell
GLUT2 vesicles
PKC βII
+
+
maltose
IM
Ca^{2+}
glucose
GLUT2
glucose
2Na$^+$
SGLT1
2Na$^+$
glucose
PKC βII
microfilaments
terminal web
tight junction

(Kellett 2001)

Fig. 1.2. (a) Glucose transport with a low glucose concentration in the lumen (before a meal), mediated by SGLT1. **(b)** is Glucose transport with a high glucose concentration in the lumen (after a meal), mediated by GLUT2. IM – isomaltose.

GLUT2 absorption, while the smaller arrow represents SGLT1 absorption. As can be seen, GLUT2 increases with glucose concentration.

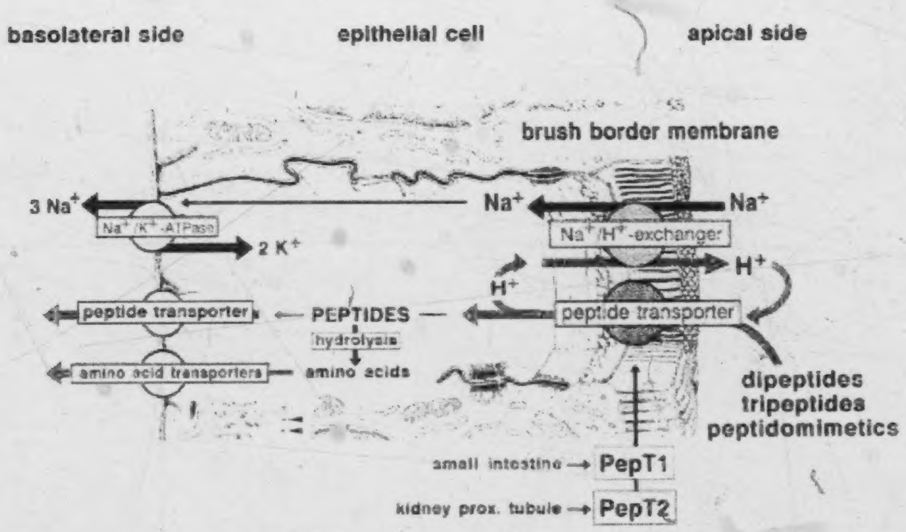
SGLT1 mediates secondary active absorption (Kellett, 2001). SGLT1 was described by Kellett (2001) as a scavenger, a transporter and a regulator. It is a scavenger because it recovers any glucose molecules that escape from the absorptive cells into the lumen at a luminal concentration less than that of blood glucose. This is the one situation in which SGLT1 can use its ability to transport glucose against the concentration gradient. At intermediate luminal concentration, where the active component exceeds the GLUT2-mediated passive component, SGLT1 has a second established role as a major transporter, but is operating in a passive mode. When the lumen of the intestine is empty before a meal, the level of GLUT2 at the brush-border membrane is low (Kellett and Helliwell, 2000). This shows how SGLT1 can regulate the onset of the activation of PKCβII so that absorption is matched to the dietary intake within minutes (Kellett, 2001).

1.3.2.4.2 Peptide Transporters

The major peptide transporter located in the small intestine of cattle is PEPT1 (Chen *et al.*, 1999). PEPT1 belongs to the peptide transporter family found in animals (Steiner *et al.*, 1995). Chen *et al.* (1999) showed, through Northern blot analysis, that the abundance of PEPT1 was higher in the jejunum and ileum, and that there was lower expression in the duodenum. Chen *et al.* (1999) sought to verify the existence of peptide transporter mRNA in the sheep forestomach and to survey the distribution of the peptide transporter mRNA in other tissues in sheep, dairy cows, pigs, and chickens. Sheep omasal epithelium mRNA was used as a template for reverse transcriptase PCR to develop a probe for Northern blot analysis. The analysis showed that PEPT1 mRNA was located in the duodenum, jejunum, and ileum of dairy cows. The major amino acid absorption site located in the small intestine of cattle was noted to be in the ileum (Chen *et al.* 1999; Wilson and Webb, 1990). PEPT1 was not located in the abomasum, liver (although it is found in the liver of humans), kidney, cecum, colon, longissimus muscle, semitendinous muscles, or mammary glands. The expression of PEPT1 mRNA is not detectable in the crypts of the small intestine. Its expression appears at the crypt-villus junction, and increases as it reaches the tip of the villi (Leibach and Ganapathy, 1996). PepT2 is another peptide transporter with similar conformation to PEPT1, except it is located in the kidney (Daniel, 1996).

PEPT1 is responsible for mediating the cellular uptake of di and tripeptides, and is distinct from the transporters that regulate the uptake of free amino acids (Amasheh *et al.*, 1997; Daniel, 1996; Doring *et al.*, 1997; Leibach and Ganapathy, 1996). PEPT1 will not transport peptides containing more than three amino acids. This particular transporter is responsible for accepting more than 400 dipeptides and over 8000 tripeptides (Doring *et al.*, 1997). PEPT1 can also transport selected β-lactam antibiotics (i.e. penicillins) (Boll *et al.*, 1994). The transporter transfers these if they have the same conformation as the peptides that it specializes in carrying. PEPT1 also plays a role in the uptake of peptides in human studies with people that have poor absorption, in order to replace the use of free amino acids. Short chained peptides, such as those capable of being absorbed by the PEPT1 transporter, are being used as substitutes for free amino acids in enteral and parenteral (introduced in areas other than the small intestine) solutions (Grimble, 1994; Leibach and Ganapathy, 1996). Enteral solutions are designed to pass through the stomach and be absorbed within the small intestine. The primary purpose of an enteral solution is to provide nutrients to those who have poor absorptive ability within the intestine. This is as opposed to using free amino acids, in the hope that absorption will occur by the respective transporters as well as those amino acids that are unstable or rarely soluble in their free form (Leibach and Ganapthy, 1996).

Figure 1.3 is a diagram from Daniel (1996) shows the transport of peptides in the small intestine. Dipeptides, tripeptides, and peptidomimetics are carried into the small intestine at the brush border membrane by PEPT1 with the aid of a proton which facilitates diffusion.


basolateral side
epithelial cell
apical side
brush border membrane
3 Na+
Na+/K+-ATPase
2 K+
Na+
Na+
Na+/H+-exchanger
H+
H+
peptide transporter
PEPTIDES
peptide transporter
hydrolysis
amino acid transporters
amino acids
dipeptides
tripeptides
peptidomimetics
small intestine → PepT1
kidney prox. tubule → PepT2

(Daniel 1996)

Fig. 1.3. Peptide absorption in the small intestine.

The H+ dependant transport is stimulated by an inside negative charge and inhibited by an inside positive charge. The transport process is thus electrogenic and independent of

Na^+, Cl^-, and Ca^{2+} (Pan *et al.*, 2001). Once through the brush border membrane, the di and tripeptides are either hydrolyzed into amino acids or kept intact. The peptides and amino acids are then transported out of the small intestine by other peptide transporters and amino acid transporters, mediated by a sodium/potassium pump.

Amasheh *et al.* (1997) found that the affinity for dipeptide interaction with a substrate binding site is affected by H+ binding to PEPT1 and by changes in membrane potential. This substrate affinity is much more pronounced when changes to pH and the Vm (in mV) are made in the case of charged substrates rather than zwitterionic substrates. Zwitterionic substrates are ionic substrates that can carry positive and negative charges at the same time. The neutral species of a charged dipeptide displayed an increased affinity for interaction with PEPT1, thus increasing the maximal transport rate (Amasheh *et al.* 1997, Pan *et al.* 2001). This implies that the neutral dipeptides will be taken up by PEPT1 faster than charged dipeptides, because they have a higher attraction to PEPT1. Pan *et al.* (2001) showed that pH affects the transport process of charged peptides. The purpose of the study by Pan *et al.* (2001) was to clone and express the ovine transporter (oPEPT1) and to characterize the function of this transporter in vitro by expression in Xenopus oocytes. It was shown that Zwitterionic and anionic substrates were transported via oPEPT1 at an optimum pH level of 5.0, while the cationic substrate was transported at an optimum level of 7.0. However, at a pH of 5.5, the cationic substrate that was used (Lys-Lys) still caused inward currents. This implies that at the physiological pH in the intestine of ruminants (4.5-5.0) oPEPT1 can transport all peptide substrates regardless of their charge (Pan *et al.*, 2001).

Peptide absorption is at its maximum at the time of birth of a mammal and decreases with age (Leibach and Ganapathy, 1996). Peptide digestion in the small intestine can be altered by diet. For example, a high protein diet increases the intestine's ability to absorb peptides (Lis *et al.* 1972).

1.4 Nutritional Requirements of Cattle

Nutrition refers to the process where an animal consumes and digests feed components for specific physiological processes (i.e. promoting growth, milk, fibre production, or to replace worn tissue) (Kellems and Church, 2002). Every ingredient in a feed ration carries a nutritive value, however if too much of one is consumed it can have an anti-nutritive effect e.g. increase in fat deposition (Ferrell and Jenkins, 1998). Different ingredients in the feed provide different nutritive factors such as energy, protein, fats, minerals, and vitamins. Because this thesis deals with the digestion and transport of glucose (which enters the digestive tract linked to other glucose molecules as carbohydrates) and peptides (which enter the digestive tract as proteins) this next section will concentrate on the nutritive and digestive factors involving carbohydrates and proteins, and make mention of fats, minerals, and vitamins. Energy comes in the form of carbohydrates, which are important because they provide the animal with the energy to produce milk, sustain body weight, sustain pregnancy, or grow. Carbohydrates are composed of carbon, hydrogen, and oxygen groups and with the general formula $C_n(H_2O)_n$. The simplest form of a carbohydrate is a monosaccharide (sugar), and the most common monosaccharides are glucose and fructose, which are shown in figure 1.4a and 1.4b respectively, in their linear and carbon ring form.

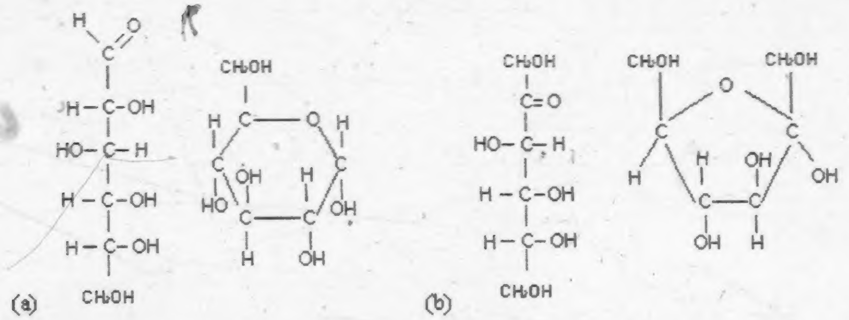


Fig. 1.4. (a) Linear form and carbon ring structure of glucose (b) Linear form and carbon ring structure of fructose.

Although these are the simplest form of carbohydrate they are the most abundant form found throughout the body.

The two major carbohydrates found in plants, and therefore in feed, are starch and cellulose (Fig. 1.5a and 1.5b respectively). These are polysaccharides, meaning they are composed of many glucose molecules linked together by glycosidic bonds. Glycosidic bonds are bonds formed between 2 monosaccharides at any of the hydroxyl groups. Starch is approximately 700g/kg of the dry matter found in cereal grains (Russel and Gahr 2000). Cellulose is the most abundant organic compound and requires microbial enzymes (i.e. rumen microbes) to convert it to absorbable forms of energy (Russel and Gahr 2000). These dietary carbohydrates can be digested in the rumen, but any starch that escapes fermentation must be converted to single sugars (i.e. monosaccharides) in order to be absorbed within the small intestine of ruminants (Kellems and Church, 2002).

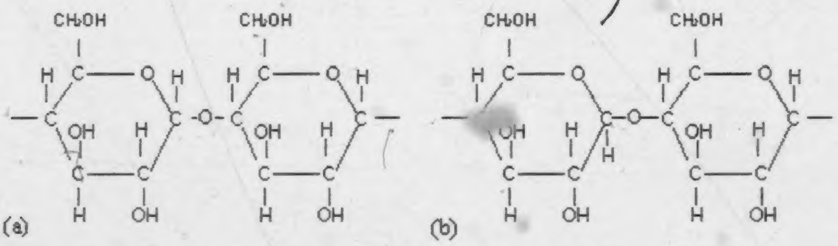


Fig. 1.2 Carbon structures of starch (a) and cellulose (b).

When it comes to carbohydrate digestion, the bacteria found in the rumen perform most of the fermentation (Huntington, 1997). This fermentation creates volatile fatty acids, which are acetic, propionic, and butyric acids, as well as CO_2, methane, and heat. The volatile fatty acids are absorbed by the epithelial surface of the rumen (Ruckebusch *et al.*, 1991). A typical molar ratio of acetate:propionate:butyrate is 66:20:14 (Russel and Gahr, 2000). Diets that are high in concentrate (high grain diets) pass through the rumen faster than those high in forage (Kellems and Church, 2002), and also tend to produce more propionate than acetate when fermented in the rumen, thus decreasing the amount of methane produced (Russel and Gahr, 2000). Propionate is the only VFA that provides a major source of carbon for gluconeogenesis (glucose production), thus it is important to promote its production within the rumen (Russel and Gahr, 2000). It is important to reiterate the fact that efficiency of use of VFAs throughout the body of a cow is less than if the carbohydrates were digested and absorbed directly in the small intestine (Kellems and Church, 2002), because the absorptive capabilities of the small intestine forms part of the base of this thesis.

Proteins are long chains of amino acids. They are broken down in the gastrointestinal tract of a cow to peptides and single amino acids. Peptides can range from di and tri peptides (2 and 3 linked amino acids respectively) to polypeptides, which are longer, chained peptides. Most proteins that are found in plants and animals are made up of combinations of 20 amino acids, joined together in different orders. These 20 amino acids are made up of essential and nonessential amino acids. Essential amino acids are those in which the animal's own metabolism cannot produce enough of, or at all in most cases, and which must be provided in feed. The nonessential amino acids are those, which can be produced in sufficient amounts by the animal. The microbes in the rumen and large intestine of cattle produce microbial protein from non-protein nitrogen supplied by the diet. Microbial protein consists of the essential and nonessential amino acids, which can be utilized by the body of the cow and therefore provide the necessary amino acids (Church and Pond, 1988).

Lipids, also known as fats, provide energy and essential fatty acids to the animal. There are four different types of lipids: glycolipids, lipoproteins, phospholipids, and sterols. Glycolipids are essential for normal cellular processes and are made up of lipids and carbohydrates. Lipoproteins are a complex of lipids and apolipoproteins, which is the form in which lipids are transported through the blood stream. Lipoproteins have different densities: high-, low- and very-low. The amount of lipid within the lipoprotein determines these densities. Phospholipids contain phosphorus and fatty acids and are a part of cellular membranes. Finally sterols are important for maintaining essential functions (vitamins A,D,E,K, and cholesterol).

Minerals are inorganic components found in the diet. There are two different types of minerals: macrominerals, and microminerals. Macrominerals are found in concentrations in the body in excess of 100ppm and include Ca, P, Cl, Mg, Na, and S. Microminerals are found in concentrations in the body of less than 100ppm and include Cr, Co, Cu, F, Fe, I, Mn, Mo, Ni, Se, Si, and Zn.

Vitamins are required by cattle in small amounts, and are essential for their tissues. There are two types of vitamins: water-soluble and fat-soluble. The fat-soluble vitamins were mentioned earlier, while the water soluble vitamins consist of vitamin C and the B-complex vitamins. The water-soluble vitamins are used as cofactors for enzymes. The fat-soluble vitamins are necessary for body functions.

1.5 Gene Expression

The field of genomics has evolved over the years from mainly structural genomics to a greater emphasis on functional genomics. Structural genomics is concerned with what the genome looks like, while functional genomics deals with how the genome works. Although more and more genes are being identified and sequenced, the function of the majority is unknown. The purpose of functional genomics is to determine what the gene does, or if that is known, why it is expressed at certain times. These questions, once answered, can assist in tackling many different and difficult problems related to the organism from which they were derived. For example, cattle eat in order to sustain body weight, to grow, and sustain pregnancy. How much they eat affects the amount of money the producer spends on feed. It is not known by the producer, how much the individual cattle actually need to sustain themselves. Different cattle require different levels of nutrition. It is the hypothesis of this thesis that cattle with a higher expression of these transporters and a lower expression of ghrelin, will require less feed. If cattle could be selected to have a certain expression level of these genes it could assist in a selection index for cattle. The genes, however, must first be identified in the gastrointestinal tract. The method used for detection in this thesis is the real time polymerase chain reaction (PCR).

Realtime PCR is based on the detection of a fluorescent signal produced during the amplification of a PCR product (Grove 1999). A probe known as a TaqMan MGB probe is designed to target the sequence between the forward and reverse primers. This probe is labeled with a reporter fluorochrome (which fluoresces when it is excited) at the 5 prime end and a quencher fluorochrome (stops fluorescence by the reporter) at the 3 prime end. The probe has a higher T_m than the primers. Thus, during the extension phase, the probe must be 100% hybridized for success of the assay (Grove 1999). The probe is degraded by the nuclease activity of Taq, releasing the reporter fluorochrome, as the Taq polymerase extends the primer. The amount of product generated in each cycle is reflected by the amount of fluorescence released during amplification (Grove 1999). This technique is very specific, as it needs three oligos to anneal to the DNA before data can be collected (Grove 1999).

A housekeeping gene that is expressed at the same level all of the time is used to compare the genes studied in the experiment to see if there is a relative change in expression of that particular gene, under different circumstances.

Kölle *et al.* (2001) used real time RT-PCR (RT refers to reverse transcriptase), among other methods, to determine whether GH and GHR were expressed in bovine preimplantation embryos, as well as localize GH, GHR, and their transcripts during early embryonic development and to demonstrate GH-responsive functions during in vitro embryogenesis. The real time PCR was used to quantitate developmental changes in GHR mRNA abundance. The housekeeping gene used in this experiment was GAPDH (glyceraldehyde phosphate dehydrogenase). They determined that GHR mRNA synthesis strongly increases from a Day 2 to a Day 6 embryo. Day 6 had the highest level of synthesis of the 7-8 day standard culture.

Mazerbourg *et al.* (2001) used real time PCR to determine whether PAPP-P (pregnancy-associated plasma protein-A) was involved with insulin like growth factor binding protein-4 (IGFBP-4) proteolytic degradation of preovulatory ovarian follicles in ovine, bovine, porcine, equine, and human species. IGF is responsible for amplifying gonadotropin action in the ovary. The real time PCR showed that there was a correlation between IGFBP-4 and PAPP-A RNAs. It also showed that PAPP-A mRNA was at its highest expression in granulosa cells from bovine and porcine fully differentiated follicles, when compared to immature and atretic follicles. IGFBP-4 increases with follicle growth (Besnard *et al.*, 1997). Thus Mazerbourg *et al.* (2001) proved that PAPP-A was involved in the IGFBP-4 degradation in ovine, bovine, porcine, and equine preovulatory follicles.

1.6 Epithelial Transport Measurement

Secretory and absorptive epithelia are both situated such that the basal lamina is on the side of the cell which faces the blood capillaries (Davison, 1989). The junctional complexes are at the apical end of the cell, thus secretion and absorption occur in opposite directions with the cell. Secretion involves the transport into the cell across the basolateral membrane, followed by the exit across the apical membrane. Solutes that are being absorbed have to cross the apical membrane first, followed by the basolateral membrane.

Ussing chambers are used to measure transport across epithelial tissue. In the 1940s and 1950s Hans Ussing and colleagues conducted experiments to explain the mechanism of NaCl transport across isolated frog skin. During this time an experimental protocol for studying epithelial ion transport was developed. "This technique which involves 'short-circuiting" and the use of radioactive tracers to measure ion fluxes is now used by gastrointestinal physiologists to measure secretion and absorption" (Davison, 1989).

The Ussing Chamber clamps the isolated epithelial tissue (from now on tissue will represent epithelial tissue) so that both sides are bathed in a solution of a known composition. The electrical potential difference across the tissue is held at zero by voltage clamps. This is done through "short-circuiting", i.e. passing a current through an external circuit connected between the electrodes at the two ends of the chamber. This cancels out the potential difference (V_m) created by the tissue. These electrical conditions, along with both sides of the tissue bathed in the same solution, create no electrochemical gradient across the tissue thus any net ion movement is caused by active transport. The current that passes through the external circuit to keep the V_m at zero ('short-circuiting current'; I_{sc}) is used to measure the total flux of ionic charge across the tissue in the absence of electrochemical gradients.

By using radioactively labeled material the unidirectional fluxes of ions across the tissue, i.e. the rate of appearance of the labeled material on the opposite side of the tissue indicates unidirectional flux. This unidirectional flux can be indicative of the transport activity of specific nutrient transporters, i.e. glucose.

1.7 Objectives

The objective of this thesis is to determine if the level of glucose transport determined using Ussing chamber experiments or the levels of expression of particular glucose or peptide transporters (GLUT2, SGLT1, and PEPT1), as well as an appetite stimulating peptide (ghrelin) located within the duodenal and jejunal tissue are related to feed efficiency in cattle, more specifically residual feed intake. To this end animals determined to be at either extreme levels of RFI were used.

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2.0 mRNA Expression of Duodenal GLUT2, SGLT1, PEPT1, and Ghrelin in Relation to Animals with High and Low RFI Values

2.1 Introduction

How cattle transport nutrients and factors that affect their DMI could have a major impact on their residual feed intake (RFI). The overall expression of mRNA corresponding to glucose transporters GLUT2 and SGLT1, peptide transporter PEPT1, and the appetite stimulating peptide ghrelin could be genes that are responsible for differences in RFI found in cattle.

The objectives of this experiment were to determine whether steers that exhibit the extreme levels of RFI (positive and negative) differentially express mRNA representative of the nutrient transporter genes: GLUT2, PEPT1, SGLT1, as well as ghrelin within the duodenum. The null hypothesis for each of these genes was as follows: there is no difference in mRNA expression of the gene (GLUT2, PEPT1, SGLT1, and Ghrelin) within the duodenum between steers with high and low RFI ($\alpha=0.05$).

2.2 Materials and Methods

2.2.1 Tissue Samples

Duodenal samples were taken from the steers in the study by Basarab *et al.* (2003). Basarab *et al.* (2003) examined the interactive effects between residual feed intake and body composition in young beef cattle. The residual feed intake values calculated for this experiment were done so by Basarab *et al.* (2003). Samples were collected from all of the steers at the slaughter facility located in Lacombe, Alberta. Once collected, the tissues were frozen in liquid nitrogen to be transported back to the laboratory in Edmonton where they were stored at –80°C. The duodenal samples consist of scrapings of the mucosal lining. Duodenal samples from 40 steers with high RFI (20) and low RFI (20) values were taken from each year and used in this experiment. A list of the steers used, their slaughter years, line (M1, M2, M3, M4, TX), age of dam, day 0 weight (weight at beginning of trial), and RFI is provided in Table 2.1. The base breeds for the Beefbooster lines are as follows: M1 is Angus, M2 is Hereford, M3 is represented by various small breeds, M4 is Limousin and Gelbvieh, and finally the base breed for the TX line is Charolais. RNA was extracted from the samples (see section 7.1 of appendices), a dnase treatment was then applied (section 7.2), finally the samples were reverse transcribed to cDNA (section 7.3). Once reverse transcribe the samples were then put through a realtime polymerase chain reaction involving primers and probes designed for specific sequences of the particular genes of interest (sections 7.4 and 7.5).

2.2.2 Statistical Analysis

Data acquisition during the reaction is by fluorescence resonance energy transfer (FRET). The threshold cycle or Ct value is the cycle at which a statistically significant increase in the magnitude of the fluorescent signal generated by the given set of PCR conditions (ΔRn) is first detected. The Ct value occurs when the sequence detection system begins to detect the increase in fluorescent signal associated with an exponential growth of PCR product. This means that in order to get an accurate measurement, the threshold level is set where the signal first begins to become linear.

The PROC GLM procedure was to test if there was a significant difference in expression between animals that had positive and negative RFI. Before these analysis methods can be applied the data must be corrected for by cyclophilin (see section 7.6). If there were any significant changes in expression, a difference in fold change was calculated using the procedure found in section 7.7.

2.2.2.1 mRNA Expression Analysis

Data analyzed using the GLM procedure of SAS (SAS Inst. Inc., 1996) carried the following model:

$$Y_{ijklmn} = \mu + R_i + A_j + S_k + D_l + W_m + \varepsilon_{ijklmn}$$

Where Y_{ijklmn} is the level of the mRNA expression as an indicator of the expression of each gene of interest expressed (ΔCt), R_i represents the fixed effect of the RFI group (steers with a negative RFI; steers with a positive RFI), A_j is the fixed effect of year (i.e. the year the animal was slaughtered 2000 or 2001), and S_k represents the fixed effect of the steer's line (M1, M2, M3, M4, or TX). D_l and W_m are covariates and represent the age of the dam and the day zero weight for each steer. Finally ε_{ijklmn} is the residual error of the model. All F-tests were carried out using Type III sums of squares. Multiple comparisons of the level of expression of the gene of interest for the RFI group were performed by the least squares method.

Correlations between the mRNA expression of each of the genes as well as between each of the genes and the actual RFI values were also tested.

2.3 Results

The results from the mRNA expression analysis of the different genes of interest with respect to residual feed intake group can be found in table 2.2. There did not appear to be any significant differences in the mRNA expression of any of the genes of interest between the different residual feed intake groups. Line did not appear to have a significant effect on the expression of the transport genes. However there does appear to be a significant difference in the expression of ghrelin mRNA between the different lines, in particular between the TX line and the M1, M2, and M4 lines (Table 2.3). Using the fold change equation found in section 7.7. Ghrelin was expressed 8.2, 10.3, and 15.3 times higher within the duodenum of the M1, M2, and M4 lines than in TX line, respectively. Also there is a significant difference in expression between the M4 and M3 lines. The M4 line expressed ghrelin within the duodenum 6.1 times higher than M3 line. The effect of year was only found to be significantly different in the expression of SGLT1 where the animals from the 2001 portion of the study had an expression level 2.2 times higher than those in 2000 portion of the study. The covariate age of the dam appeared to have no effect on the mRNA expression of any of the genes of interest. The covariate day zero weight appeared to have an effect only on the expression of ghrelin.

Finally significant correlations were found between GLUT2 and SGLT1 (P=0.0049; r=0.44) and between GLUT2 and ghrelin (P=0.0060; r=0.43), figures 2.1 and 2.2 respectively.

2.4 Discussion

There are a number of possible explanations for the fact that no significant differences were found in the expression of mRNA as an indicator of these genes between the two

RFI groups. For example, the steers were not fed while at the slaughter facility and thus the expression of these genes may therefore be lower. The mRNA expression of the genes within the duodenum would be determined by the amount of digesta remaining in the gastrointestinal tract. Because the steers were not fed while at the slaughter facility the nutrient transporters would be in somewhat of a resting state because there is very little need for them. GLUT2 in particular would be non-functioning because as stated in chapter one, it is induced via recruitment by SGLT1, which is caused by an influx of nutrients into the small intestine (i.e. eating), although it does take some time before a ruminant to reach a truly post absorptive state, because of the size and the capacity of the rumen. It is also important to note that the standard error of the mean for GLUT2 is very high, and could be due to the great deal of variation in its expression (i.e. different stages of glucose absorption) between the animals within the positive and negative RFI groups. PEPT1 is in a similar situation as the other transporters because if there are no, or limited amounts, of di and tripeptides to transport then it too would be considered in somewhat of a resting state. Ghrelin on the other hand may not be in a resting state. Because levels of plasma ghrelin were found to be high in animals before feeding (Hayashida *et al.*, 2001; Murakami *et al.*, 2002; Sugino *et al.*, 2002), it could thus be conceived that these animals would have high levels of ghrelin mRNA expressed within the duodenum.

If the tissue samples had been treated with a nutrient mixture (peptide or glucose rich) before freezing, there is a possibility that this mixture of nutrients would have affected the expression of these genes. By treating the tissues with a solution containing a high concentration of nutrients one might simulate what could be considered normal digestive conditions within the small intestine of a live animal. This would, in turn, create "normal" transport conditions within the small intestine and thus demonstrate the expression of the mRNA of the genes of interest as would be found in a live animal. At the same time, it would also normalize the tissues between the animals to one another. By treating them all with the same solutions one would remove some of the external variables affecting expression, such as, for example, the amount of digesta that remained within the small intestine from before the animals were shipped. Ghrelin would also be influenced because of how it is affected by intake, and thus if one animal were to have more digesta within its duodenum than another, this would affect the expression of ghrelin.

Duodenal tissue was the only small intestinal tissue collected from each animal during the study providing samples for this experiment. The expression of the transporters SGLT1 and PEPT1 has been found to be highest in jejunal tissue (SGLT1 Zhao *et al.*, 1998; PEPT1 Chen *et al.*, 1999). It is thought that the ileum serves as a compensatory tissue, which absorbs the left-over glucose that was not absorbed by the duodenum and jejunum (Ferraris and Diamond 1986). In other words the majority of absorption occurs within the duodenal and jejunal tissues. Ghrelin mRNA has been shown to be more highly expressed in the duodenum, but it is still expressed within the jejunum (chapter 3; Hosoda *et al.*, 2000), thus both tissues should be studied in the future for all of the genes of interest.

In terms of the differential expression of ghrelin between the different lines, it would be conceivable to consider the fact that differences in the expression of ghrelin would exist between the different lines. However it is inaccurate to draw any conclusions from this output because of the small representation of each line within the model. More animals would be needed in order to determine with any confidence that there were significant differences in expression between the different lines.

Finally it is interesting to point out that there are significant moderate correlations between GLUT2 and SGLT1, and between GLUT2 and ghrelin. The positive correlation between GLUT2 and SGLT1 (r=0.44) means that when an animal has a high expression of GLUT2 it will have a high expression of SGLT1 which would be understandable in terms of glucose transport and absorption. The theory is that SGLT1 recruits GLUT2 to aid in glucose absorption (Kellett, 2001), so it is conceivable to infer that if the expression of SGLT1 is high, so then would be the expression of GLUT2. In terms of the positive correlation between GLUT2 and ghrelin (r=0.43), it can be concluded from these results that if a steer has a high expression of ghrelin within its duodenum, the expression of GLUT2 will also be high. It is difficult to conclude anything further from these results, because there is nothing in the literature that compares the expression of these two genes, or anything involving these two genes together for that matter.

In order to be able to compare the overall expression of the mRNA of the different genes of interest, it will be important to consider the points from the above section:

1. Treating the tissue with a nutrient solution before freezing it
2. Use the jejunum

By considering these options, it will aid in the comparison of the differences in mRNA expression of the particular genes of interest between the RFI groups.

Table 2.1. The kill number, slaughter year, line, age of dam, day 0 weight and RFI of the 40 steers.

Kill #	Slaughter Year	Line	Dam Age	Day 0 Wt (kg)	RFI (kg)
202	2000	M1	4	345.2	1.01
203	2000	M3	6	253.9	1.16
211	2000	M1	3	378.7	1.27
214	2000	M1	4	437.3	-0.75
306	2000	M1	3	340.3	0.88
310	2000	M2	10	288.0	-1.15
401	2000	M1	6	369.6	1.19
402	2000	M4	3	313.4	-1.02
405	2000	M3	12	308.8	-0.93
407	2000	TX	4	281.9	-1.27
408	2000	M1	10	291.0	1.01
410	2000	M2	4	294.1	-1.28
415	2000	M4	3	352.2	-1.05
501	2000	M1	6	341.9	1.17
507	2000	TX	9	318.5	1.02
509	2000	M3	2	337.9	-0.99
510	2000	M4	3	323.8	-0.87
514	2000	TX	5	348.2	1.07
602	2000	M2	3	348.5	-1.72
614	2000	M3	7	257.1	1.01
201	2001	TX	6	369.3	-1.56
213	2001	M3	11	359.9	0.62
305	2001	TX	4	324.7	-1.18
306	2001	M3	3	360.2	0.97
310	2001	M2	4	426.8	1.1
311	2001	M4	8	425.5	-1.59
401	2001	M2	4	354.8	-0.75
406	2001	M3	2	281.7	0.86
408	2001	M2	8	423.2	1.47
411	2001	M4	3	234.6	-1.11
413	2001	M1	3	351.7	1.63
504	2001	M3	2	341.9	-1.42
507	2001	M4	8	311.3	1.97
601	2001	M2	6	366.8	-1.19
603	2001	M4	6	386.6	-0.93
607	2001	M1	3	352.5	1.36
611	2001	M4	4	354.3	0.82
612	2001	M4	6	304.6	0.97
613	2001	M3	7	323.1	-1.09
615	2001	TX	3	291.7	-0.82

Table 2.2. Least squares means values of mRNA expression of genes of interest between different RFI groups.

Gene of Interest	RFI Group	ΔCt (±SEM)
GLUT2	Negative	0.38 ± 0.29^{a}
GLUT2	Positive	-0.16 ± 0.31^{a}
SGLT1	Negative	-2.10 ± 0.20^{a}
SGLT1	Positive	-1.85 ± 0.21^{a}
PEPT1	Negative	-2.40 ± 0.53^{a}
PEPT1	Positive	-2.33 ± 0.56^{a}
Ghrelin	Negative	-2.79 ± 0.17^{a}
Ghrelin	Positive	-3.49 ± 0.18^{a}

a-b values with the same gene of interest between different RFI groups with different letters differ ($P<0.05$)

Table 2.3. Least squares means of mRNA expression of genes of interest between different lines.

	Gene of Interest (ΔCt ± SEM)			
Line	GLUT2	SGLT1	PEPT1	Ghrelin
M1	0.40 ± 0.49^{a}	-1.99 ± 0.35^{a}	-2.39 ± 0.30^{a}	-3.84 ± 0.90^{a}
M2	-0.25 ± 0.48^{a}	-1.82 ± 0.34^{a}	-2.32 ± 0.29^{a}	-4.16 ± 0.88^{ab}
M3	-0.19 ± 0.43^{a}	-2.43 ± 0.30^{a}	-2.29 ± 0.26^{a}	-2.14 ± 0.78^{abc}
M4	-0.02 ± 0.42^{a}	-1.61 ± 0.30^{a}	-2.23 ± 0.25^{a}	-4.74 ± 0.77^{abd}
TX	0.62 ± 0.51^{a}	-2.04 ± 0.36^{a}	-2.59 ± 0.30^{a}	-0.80 ± 0.93^{ce}

a-e Values within the same column with different letters differ ($P<0.05$)

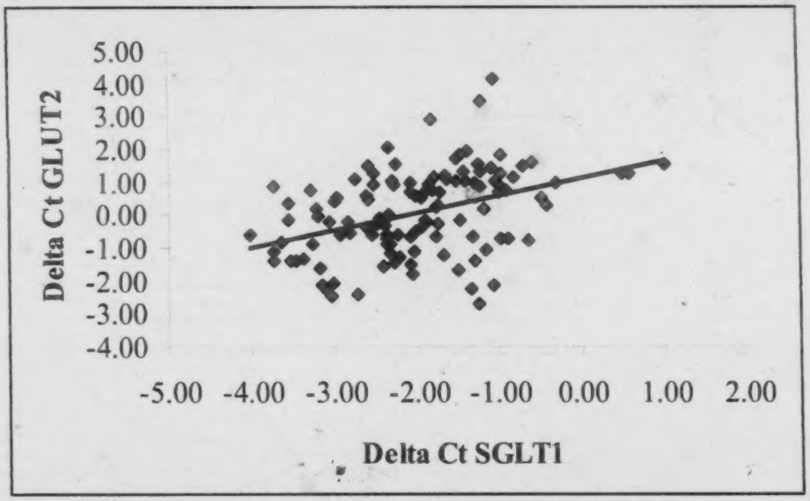

Fig 2.1. The expression of GLUT2 vs. the expression of SGLT1 (r=0.44).

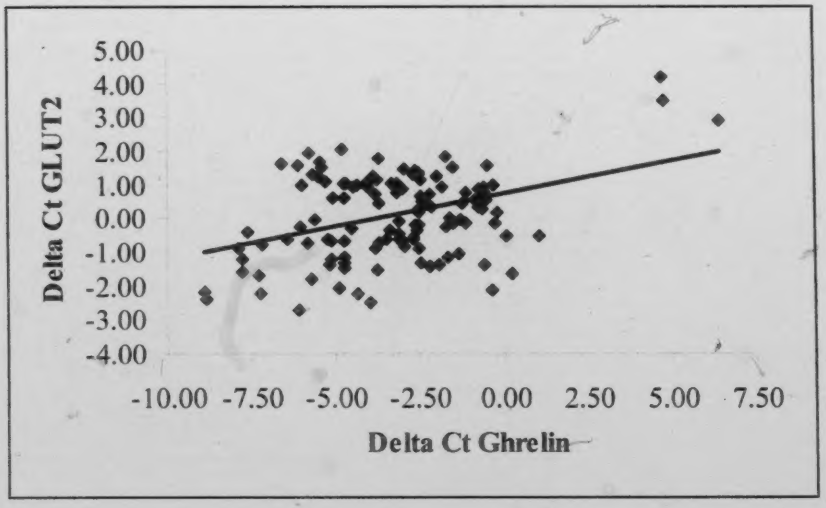

Fig 2.2. The expression of GLUT2 vs. the expression of ghrelin (r=0.43).

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3.0 Short Study: Evaluation of the distribution of ghrelin mRNA throughout the bovine gastrointestinal tract using real-time PCR

3.1 Introduction

Ghrelin has an appetite stimulating effect (Tschöp *et al.*, 2000; Nakazato *et al.*, 2001; Shintani *et al.*, 2001; Wren *et al.*, 2000; Wren *et al.*, 2001; Hayashida *et al.*, 2001). Because it has been shown that levels of ghrelin in the blood plasma have been shown to be controlled by intake (Hayashida *et al.*, 2001; Murakami *et al.*, 2002; Sugino *et al.*, 2002), it is important to examine where this signal could originate from in cattle. In rats, ghrelin is primarily expressed in the mucosal layer of the stomach, but expression has also been found in the duodenum, jejunum, ileum, and colon, in order of significance, respectively (Hosoda *et al.*, 2000). However, there are no studies within the literature that discuss the distribution of ghrelin mRNA throughout the bovine gastrointestinal tract. This information is essential to this thesis in order to determine if the tissues being studied are relevant when it comes to the gene expression of ghrelin. The purpose of this study was to determine the distribution of ghrelin mRNA throughout the bovine gastrointestinal tract.

3.2 Materials and Methods

3.2.1 Tissues

Tissues were harvested during processing of young growing beef steers, frozen with liquid nitrogen, and stored at –80°C (from the study by Basarab *et al.*, 2003). The following gastrointestinal tract tissues were used in this experiment (the number of animals supplying each tissue is given in brackets): reticulum (2), rumen (2), omasum (2), abomasum (4), duodenum (4), jejunum (2), and ileum (1). The experiment was broken down into the upper and lower GI tract (UGIT and LGIT, respectively) since the animals that the reticulum, rumen and omasum tissues were collected from were different from those that supplied the jejunum and ileum samples. Therefore the UGIT includes the reticulum, rumen, omasum, abomasum, and duodenum, and the LGIT contains the abomasum, duodenum, jejunum, and ileum. All of the animals had duodenal and abomasal tissue collected and these samples were used for comparison of the upper and lower tracts. Total RNA was isolated from each of the tissue samples and then prepared for analysis using the realtime PCR, as done in chapter 2.

3.2.2 Statistical Analysis

Data was analyzed, for the UGIT and LGIT respectively, by using the Analysis of Variance Procedure of SAS (SAS Inst. Inc., 1996) using the model:

$$Y_{ijk} = \mu + T_i + A_j + \varepsilon_{ijk}$$

where Y_{ijk} is the level of ghrelin expression (ΔCt) of tissue i of animal j of the particular tissue of interest, while μ is the overall mean of the tissue sample, T_i represents the fixed effect of tissue, A_j is the fixed effect of animal, and ε_{ijk} is the residual error of the model. The residual error was used as the error term to test for the fixed effect of tissue. All F-tests were carried out using Type III sums of squares. Multiple comparisons of the level of ghrelin expression among tissues were performed by the least squares method.

3.3 Results and Discussion

The average expression (average ΔCt), standard deviation, standard error of each tissue, as well as the fold change with respect to the tissue with the lowest level of expression (Reticulum for the UGIT; Ileum for the LGIT) is represented in Table 1. As expected, the expression levels of ghrelin between animals were significantly different ($p<0.0001$) for both the UGIT and LGIT (data not shown). In addition there were significant differences between different gastrointestinal tissues in the level of expression of ghrelin ($P < 0.0001$) for both UGIT and LGIT (Figure 1).

Significant differences between tissues in level of expression of ghrelin in the UGIT experiment were in the order abomasum>duodenum>rumen>omasum ($P < 0.0001$). There were no significant differences in ghrelin expression between the omasum and reticulum ($P > 0.10$). Significant differences between tissues in level of expression of ghrelin in the LGIT experiment were in the order, abomasum>duodenum>jejunum>ileum ($P < 0.0001$). These results imply that there is a significant difference in ghrelin expression throughout the bovine gastrointestinal tract. Because the degrees of expression between the different tissues are so vast some assumptions can be made. It can be assumed that the expression of ghrelin throughout the bovine gastrointestinal tract follows the order of in highest to lowest abomasum>duodenum>jejunum>rumen>omasum and ileum>reticulum.

The results of this study therefore show that the relative expression of ghrelin in the ruminant GI tract is similar to that in the monogastric animal (Hosoda *et al.*, 2000). Highest expression occurs in the abomasum, which most closely resembles the stomach in the monogastric followed by the duodenum and jejunum. Therefore the duodenal and jejunal tissue can be used for the experimental studies involving the expression of ghrelin within this thesis, because of the level of expression demonstrated within these tissues as compared to other tissues of the GIT.

Table 3.1. The mean expression, standard deviation, standard error for each tissue, and the fold change between tissues with the lowest expression within each group.

Tissue	Mean	Std Dev	Std Err	Fold Change compared to Reticulum	Fold Change compared to Ileum
Reticulum	10.283	0.891	0.364	1.000	
Rumen	3.475	2.075	0.847	1.121×10^2	
Omasum	8.799	1.738	0.524	2.797	
Abomasum	-7.766	0.964	0.278	2.712×10^5	3.819×10^4
Duodenum	-3.129	0.500	0.144	1.090×10^4	1.534×10^3
Jejunum	1.618	0.985	0.402		5.716×10^1
Ileum	7.455	0.681	0.278		1.000

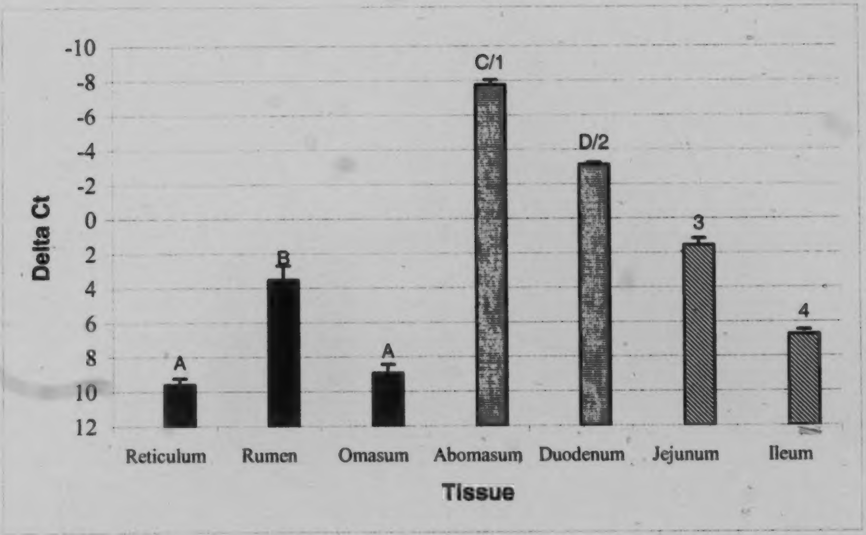

Fig. 3.1. Variation in mRNA expression (least square mean ΔCt values and standard error) between tissues in the same animal comparison. Letters represent the UGIT, while numbers represent the LGIT.
A-D Least squared means values of bars with different letters differ ($p<0.0001$)
1-4 Least squared means values of bars with different numbers differ ($p<0.0001$)

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4.0 GLUT2, SGLT1, PEPT1 and Ghrelin mRNA Expression and Functional Glucose Transport in the Bovine Duodenum and Jejunum in Response to Varying Nutrient Concentrations

4.1 Introduction

Based on the information attained from chapter 2, this chapter looks at the effects of culturing duodenal and jejunal tissue of a dairy cow in different culture solutions, to determine if the mRNA expression of nutrient transporters GLUT2, SGLT1, and PEPT1 as well as intake stimulating peptide ghrelin can be regulated. In order to regulate the mRNA expression of the particular genes of interest, different nutrient solutions must be used. Au *et al.* (2002) used a buffer solution along with 50mM glucose in order to upregulate GLUT2, as well as a 6mM glucose solution as the control. L-carnosine (β-Ala-His) is a dipeptide used to encourage PEPT1 transport (Brodin *et al.*, 2002; Ferraris *et al.*, 1988; Rajendran *et al.*, 1986; Winckler *et al.*, 1999). The purpose of using these compounds (glucose and L-carnosine) is to encourage the upregulation of the expression of the transport genes. Messenger RNA must be expressed before it can be translated to protein. Therefore, it is the hope that because these compounds have been used in studies to increase protein expression, that mRNA produced by the cell that encodes for these transporter proteins will be regulated by these compounds as well.

Ussing chambers can be used to measure actual glucose transport within epithelial tissue of the small intestine, based on the concentration of glucose normally found within the intestine of cattle, 2.0 to 6.0 mM (Okine *et al.*, 1994). The purpose of this chapter is to determine if the genes of interest and glucose transport can be regulated so that the same procedures can be used in order to establish differences in expression and transport between steers with different RFI values.

The objectives of this experiment were to determine whether duodenal and jejunal tissue samples cultured in a high nutrient culture could regulate nutrient transporter genes: GLUT2, SGLT1, PEPT1, as well as the intake stimulating peptide Ghrelin. Real-time PCR was used in order to determine the regulation of the genes of interest, also Ussing chambers were used in order to determine glucose transport of tissue at time zero and after being exposed to the high nutrient culture. The null hypothesis for each of the genes measured by the real-time PCR is as follows: there is no difference in expression of the gene (GLUT2, SGLT1, PEPT1, and Ghrelin) within/between the duodenal and jejunal tissues cultured in high, low nutrient cultures, as well at time zero (α=0.05). The null hypothesis for measuring glucose transport with the Ussing chambers is that there is no difference in glucose transport activity between duodenal and jejunal tissue cultured in a high glucose culture and at time zero (α=0.05).

4.2 Materials and Methods

4.2.1 Tissue Samples

Duodenal and jejunal samples of approximately 1 meter in length were collected from a dairy cow at slaughter. The cow was a cull from the University of Alberta Dairy Research Farm and was processed at Edmonton Custom Packers. Once the tissue was removed it was placed in a plastic bag containing cold Krebs-Ringer bicarbonate buffer, this was done for both the duodenum and jejunum. The recipe for this solution is located in the appendices (Table 6.3). The bags of tissue were kept in a cooler full of ice. It is important to note that the samples remained intact in their cylindrical form (much like a sausage), with no incisions in the lining of the intestine, except for where it was removed. The tissue remained in this solution during travel back to the university.

4.2.2 Tissue Sample Preparation

Once the tissues arrived at the university they were first flushed with cold PBS to remove any digesta that was still within the samples. They were then cut into three segments (approximately 20 cm), so they could be used in their designated point of the experiment: time zero, high glucose, high peptide low glucose (just enough glucose to keep the tissue alive and the cells functioning). The time zero tissue was cut open and a piece was used for the Ussing chambers (discussed later), also a mucosal scraping was taken and snap frozen in liquid nitrogen.

The high glucose and high peptide/low glucose tissue samples were clamped off at one side and then filled with their respective solutions (Table 6.3). The solutions were oxygenated (95% O_2 and 5% CO_2) and kept at 37°C before they were added to the tissues. Once the tissue samples were filled, they were clamped off at the other end so they looked like a sausage. These "sausages" filled with nutrient solutions were then placed in a water bath containing oxygenated Krebs-Ringer bicarbonate buffer and incubated at 37°C for one hour. After one hour had passed the tissues were removed and cut open. The duodenal and jejunal tissues that were cultured in high peptide/low glucose were immediately scraped, snap frozen, and stored in separate tubes at -80°C. As for the duodenal and jejunal tissues that were cultured in the high glucose solution; a piece of each was cut of to be used for the Ussing chambers, the remainder of the tissue was scraped, snap frozen, and stored in separate tubes at -80°C. It is important to note that each treatment within the tissues is the other's control, i.e. the high glucose is the control for the high peptide/low glucose and the high peptide/low glucose is the control for the high glucose, because it only has enough glucose to keep the cells alive.

RNA was then extracted from the frozen mucosal scrapings and the samples were prepared for analysis using the realtime PCR (same preparation as in chapter 2).

4.2.3 Ussing Chambers

Duodenal and jejunal tissue (in duplicate) were mounted in Lucite chambers exposing mucosal and serosal surfaces to 10ml of oxygenated Krebs buffer maintained at 37°C by a heated water jacket and circulated by CO_2/O_2 (5% CO_2 and 95% O_2). This was done for tissue before culturing (time zero) and after culturing in a high glucose solution (50 mM). Fructose (10mmol/L) was added to the serosal and mucosal sides. The spontaneous transepithelial potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing a short-circuit current (ISC) with an automatic voltage clamp (DVC 1000 World Precision Instruments), except for 5-10 seconds every 5 minutes when PD was measured by removing the voltage clamp. Tissue ion conductance (G) was calculated from PD and ISC according to Ohm's Law. PD is expressed as millivolts (mV), ISC as microamperes per square centimeter ($\mu A/cm^2$), and G as millisiemens/cm^2. Baseline ISC and G were measured after a 20-minute equilibration period. Increases in ISC were induced by addition of the adenylate cyclase-activating agent, forskolin (105 μmol/L), to the mucosal surface. Epithelial responsiveness was defined as the maximal increase in ISC to occur within 15 minutes of exposure to the secretagogue.

In order to measure the basal flux 5 µCi [^{3}H] 3-O-methyl-D-glucose was added to the mucosal side after mounting and the tissue was allowed to equilibrate for 15 minutes; by this time the tissue was electrically stable. Unidirectional flux (mM/cm^2/hr) from mucosal-to-serosal surface was determined for paired tissues by measuring four consecutive five-minute fluxes. The glucose flux is reported as the difference between the averaged values of the four consecutive five-minute fluxes.

4.2.4 Statistical Analysis

4.2.4.1 mRNA Expression Analysis

The PROC GLM procedure from SAS (SAS Inst. Inc., 1996) was used to determine mRNA expression. The model consists of the ΔCt value of the gene of interest, the different tissues, and the different nutrient cultures the tissues underwent:

$$Y_{ijk} = \mu + T_i + C_j + TC_{ij} + \varepsilon_{ijk}$$

Where Y_{ijk} is the level of expression (ΔCt) of the particular tissue of interest, while μ is the overall mean of the tissue sample, T_i represents the fixed effect of tissue (duodenum or jejunum), C_j represents the fixed effect of the culture the tissue was subjected to (none, high glucose, low glucose/high peptide), TC_{ij} is the fixed effect of the interaction between tissue and culture, and ε_{ijk} is the residual error of the model. It is important to note that when the effect of culture within the individual tissues was also measured and it followed the same model, but the effect of tissue as well as the interaction between tissue and culture was not included. All F-tests were carried out using Type III sums of squares.

Multiple comparisons of the level of expression of each gene of interest for the interaction between tissue and culture were performed by the least squares method. This was done in order to determine if the gene of interest was differentially expressed between the different treatment groups.

4.2.4.2 Ussing Chamber Analysis

The PROC GLM procedure was used to determine significant differences in ussing chamber results (SAS Inst. Inc., 1996). The model for the Ussing Chamber experiment consists of the following:

$$Y_{ijk} = \mu + T_i + C_j + TC_{ij} + \varepsilon_{ijk}$$

Where Y_{ijk} is the glucose flux level, PD, G, or ISC of the particular tissue of interest, while μ is the overall mean of the tissue sample, T_i represents the fixed effect of tissue (duodenum, and jejunum). C_j represents the fixed effect of culture (no culture and high glucose culture), TC_{ij} represents the fixed effect of the interaction between tissue and culture, and ε_{ij} is the residual error of the model. All F-tests were carried out using Type III sums of squares. Multiple comparisons of the level of flux, PD, G, and ISC response among the interaction between tissue and culture were performed by the least squares method.

4.3 Results

4.3.1 Realtime PCR Results for High Glucose vs. Low Glucose/High Peptide Tissues

Messenger RNA expression results for the time trial comparing the two tissues in high glucose and low glucose/high peptide cultures can be found in Table 4.1, while fold change results can be found in Tables 4.2, 4.3, 4.4, and 4.5 (GLUT2, SGLT1, PEPT1, and Ghrelin respectively).

GLUT2 was only differentially expressed within the jejunum treated with a low glucose/high peptide, with an average fold change of 3.3 times higher than the other treatments.

SGLT1 was found to be significantly differentially expressed within the duodenal and jejunal samples, however the duodenum at low glucose/high peptide was found not to be statistically significant when compared to the jejunum at high glucose. In terms of fold change the greatest fold changes occurred within the jejunum cultured in a low glucose/high peptide solution. It was found to be expressed 5.78, 3.56, and 4.99 times higher than the duodenum at low glucose/high peptide, duodenum at high glucose and the jejunum at high glucose respectively.

PEPT1 was differentially expressed between all of the treatment groups. It was found to be expressed highest within the jejunal tissues. The jejunum cultured in the low glucose/high peptide solution was found to express the transporter 2.93 and 4.50 times higher than that of the duodenum at low glucose/high peptide and high glucose, respectively. However the jejunum cultured with a high glucose solution was found to be 4.32, and 6.63 times higher than those treatment groups respectively, but only 1.47 times that of the low glucose/high peptide jejunal tissue.

Ghrelin was also differentially expressed between all of the treatment groups and was expressed as follows (from highest to lowest) duodenum low glucose/high peptide>duodenum high glucose>jejunum low glucose/high peptide>jejunum high glucose. The duodenal tissue treated with the low glucose/high peptide solution expressed ghrelin 3.07, 9.71, and 92.41 times higher than the duodenum high glucose, jejunum low glucose/high peptide, and jejunum high glucose tissues respectively. Ghrelin was expressed within the duodenal tissue cultured with a high glucose solution 3.16 and 30.06 times higher than the jejunum low glucose/high peptide, and jejunum high glucose tissues respectively. Finally the jejunum cultured with a low glucose/high peptide solution expressed ghrelin 9.51 times higher than jejunal tissue cultured in the high glucose bath.

4.3.2 Ussing Chamber Experiment

4.3.2.1 Ussing Chamber Results

The model showed that the flux level (glucose transport activity) was affected by tissue, culture, and the interaction between tissue and culture (P=0.0196). Upon further investigation it was determined that tissue, and the interaction between tissue and culture had a significant affect on glucose transport activity (P=0.0451, and P=0.0171 respectively), however the effect of culture was approaching significance (P=0.0947). The duodenal tissue in the high glucose culture had the highest level of flux and therefore had the highest level of glucose tranport activity followed by the jejunal tissue at time zero, the duodenum at time zero, and the jejunal tissue in a high glucose culture. A list of these values can be found in Table 4.6. The duodenal tissue in the high glucose culture differed significantly from the duodenum at time zero, the jejunum at time zero, and the jejunal tissue cultured in the high glucose bath (P=0.0077, P=0.0210, and P=0.0045 respectively). However none of the other tissues differed significantly from each other.

In terms of the electrical values there were no significant effects of tissue, culture, or the interaction between the two found for PD, and G. Tissue had a significant effect on ISC (P=0.0382), however culture and the interaction between tissue and culture did not. The effect of the interaction between tissue and culture can be found in table 4.6. The forskolin response was included just to determine if the tissue was still alive at the end of the experiment. Values above zero means that the tissue was still alive.

4.3.2.2 mRNA Expression Results from Ussing Chamber Tissues

The results for the expression of GLUT2 and SGLT1 within the tissues at time zero and cultured in a high glucose solution (for 60 minutes) can be found in Table 4.7, while the fold change results can be found in Tables 4.8 (GLUT2) and 4.9 (SGLT1). GLUT2 was expressed differentially within the tissues, however the duodenum and jejunum at time zero, as well as the duodenum and jejunum cultured in a high glucose solution were not significantly different between each other. GLUT2 was expressed 2.03 and 2.64 times higher within the duodenum and jejunum at time zero than duodenum treated with high glucose. It was also expressed 1.88 and 2.45 times higher than within the duodenum and jejunum at times zero than the jejunal tissue treated with high glucose.

SGLT1 was only not significantly differentially expressed between the duodenum and jejunum at time zero. It was expressed highest within the jejunum at time zero with a fold change of 1.66 and 2.33 times higher than that of the duodenum jejunum cultured with high glucose. This was followed by the expression within the duodenum at time zero being 1.52 and 2.13 times higher than the expression within the duodenum and jejunum cultured with a high glucose solution. GLUT2 within the high glucose cultured duodenum was expressed 1.40 times higher than that of the jejunum cultured in the same manner.

4.4 Discussion

The level of expression for GLUT2 and SGLT1 was highest in the jejunal tissue cultured with the low glucose/high peptide solution. This could be due to two factors. The first factor is GLUT2 and SGLT1 are found to be expressed higher in the jejunum (Cho-Leam, 1994; Zhao *et al.*, 1998) in the bovine gastrointestinal tract, and the majority of the studies reviewed have performed experiments on the jejunum when studying intestinal glucose transporters (eg. Au *et al.*, 2002; Cui *et al.*, 2003; Hirsh and Cheeseman, 1998). The second and more important factor in terms of the expression of GLUT2 is the fact that its expression has been known to decrease when exposed to glucose over a long period of time. This was demonstrated by Cho-Leam (1994). She found that, mRNA levels of duodenal and jejunal GLUT2 in rats fed a high carbohydrate diet over a seven day period, decreased. Because the bovine duodenal and jejunal tissue was exposed to a high concentration of glucose over a period of an hour, it is conceivable that the same effect could have occurred. The bovine small intestine is not accustom to such high levels of glucose. The majority of the time it has a concentration of 2 to 6mM (Okine *et al.*, 1994). A time trial could provide the necessary information to attain the optimum time required to upregulate glucose transporter mRNA.

PEPT1 results were consistent with the findings of Chen *et al.* (1999) that the expression of this particular transporter is higher in the jejunum than within the duodenum. Although the results indicate that there was a significant change in the expression of the mRNA of this particular transporter between the treatment groups of the tissues, the fold change results show that the change was not that substantial. The expression of PEPT1 was only 1.54 times higher within the high peptide duodenal sample than that which was cultured with a solution not containing a peptide.. The opposite was true for the results between the jejunal tissues with PEPT1 being expressed 1.47 times higher within the tissue cultured in a solution not containing a peptide. These fold change results show that although differences did occur, they were not substantial.

The expression of ghrelin was consistent with the results found by Hosoda *et al.* (2000), and within chapter 2, which shows that ghrelin is expressed higher within the duodenum followed by the jejunum. The results of this experiment show that glucose has a direct effect on the expression of ghrelin within these tissues. The presence of glucose appears to decrease the expression of ghrelin. This is consistent with results found by Tschöp *et al.* (2000), who showed that water distention does not affect ghrelin expression, but actual nutrients control the levels of ghrelin. The results of my experiment are also consistent with those found by Hayashida *et al.* (2001) when dealing with plasma ghrelin. They found that the levels of plasma ghrelin were higher in cattle before a meal and were reduced ($P<0.05$) one hour after feeding. These results are much like those determined by this experiment dealing with mRNA expression. Ghrelin mRNA has been found to increase with starvation stress (Asakawa *et al.*, 2001). This means that when the animal is not fed the expression of ghrelin mRNA increases. The expression of ghrelin within the small intestine after a meal could have a direct link with residual feed intake. It is conceivable that an animal with positive residual feed intake after a meal could have a higher expression of ghrelin than that of an animal with negative residual feed intake thus causing the former to eat more. By culturing the tissues with the same solutions for the same amount of time, this would "normalize" the tissue samples of each individual animal to each other, thus removing the variable factor, i.e. feed intake.

The ussing chamber flux results demonstrate that glucose transport did occur, within both tissues, upon further examination, it appears that glucose transport was upregulated within the duodenal tissue treated with the high glucose solution. It is interesting to point out that the electrical values of the two duodenal treatments were not significantly different from each other. This indicates that sodium coupled transport of glucose (SGLT1), which would cause electrical differences to occur in order to 'short-circuit' the tissue to a potential difference of 0, was not found to be statistically different. This implies that two other routes could have transported glucose. Glucose could have slipped through the tight junctions of the epithelium or it could have been transported across by GLUT2 (Cheeseman, 2002). The electrical results of the jejunum on other hand show that the jejunal tissue may have died. This cannot be certain because there were still flux levels. The mRNA results of GLUT2 and SGLT1 of this section are not unlike the previous section. The glucose transporter mRNAs were found to be expressed highest within the jejunum that had not been cultured. The same can be said for the duodenal tissue; non cultured tissue had a higher expression of mRNA of these transporters than the duodenal tissue that was cultured. Cho-Leam (1994) found that there was no correlation between the expression of GLUT2 mRNA and that of GLUT2 protein; therefore it is definitely conceivable that GLUT2 transported the glucose across the epithelial tissue.

In order to be able to accurately compare the expression of the mRNA as an indicator of these genes of interest and glucose transport capabilities between animals with high and low RFI values, two things are suggested by these results. A time trial must be conducted in order to determine a point at which glucose transporter expression can be upregulated. The hope will be that this time trial will not only suffice for the glucose transporters, but for transport measurement, PEPT1, and ghrelin as well. The second important point is that a net flux between the epithelial tissues of the duodenum and jejunum must be measured:

$$\text{Net Flux} = \text{Flux}_{ms}\ (\text{mucosal to serosal}) - \text{Flux}_{sm}\ (\text{serosal to mucosal})$$

This is done to determine whether or not glucose transport occurred or if glucose is passing through the tight junctions.

Table 4.1. mRNA expression results; mean values and comparison using LSMEANS.

Tissue	Culture	mRNA Expression levels of Genes of Interest (±SEM)			
		GLUT2	SGLT1	PEPT1	Ghrelin
Duodenum	Low glucose/high peptide	$-1.83^{ab}\pm0.18$	$-2.59^{a}\pm0.08$	$-2.59^{a}\pm0.12$	$-1.79^{a}\pm0.16$
Duodenum	High glucose	$-1.51^{b}\pm0.18$	$-3.29^{b}\pm0.08$	$-1.97^{b}\pm0.12$	$-0.17^{b}\pm0.16$
Jejunum	Low glucose/high peptide	$-3.17^{c}\pm0.18$	$-5.12^{c}\pm0.08$	$-4.14^{c}\pm0.12$	$1.49^{c}\pm0.16$
Jejunum	High glucose	$-1.13^{bd}\pm0.18$	$-2.80^{ad}\pm0.08$	$-4.70^{d}\pm0.12$	$4.74^{d}\pm0.16$

a-d cells within columns with different letters differ ($p<0.05$)

Table 4.2. GLUT2 fold change results from tissue culturing experiment (DL=duodenum low glucose/high peptide; DH=duodenum high glucose; JL=jejunum low glucose/high peptide; JH=jejunum high glucose)

	Fold change from each Treatment Group			
Tissue and Culture	DL	DH	JL	JH
DL	1.00	0.80	2.53	0.62
DH	1.25	1.00	3.16	0.77
JL	0.40	0.32	1.00	0.24
JH	1.62	1.30	4.11	1.00

Table 4.3. SGLT1 fold change results from tissue culturing experiment.

	Fold change from each Treatment Group			
Tissue and Culture	DL	DH	JL	JH
DL	1.00	1.62	5.78	1.16
DH	0.62	1.00	3.56	0.71
JL	0.17	0.28	1.00	0.20
JH	0.86	1.40	4.99	1.00

Table 4.4. PEPT1 fold change results from tissue culturing experiment.

	Fold change from each Treatment Group			
Tissue and Culture	DL	DH	JL	JH
DL	1.00	0.65	2.93	4.32
DH	1.54	1.00	4.50	6.63
JL	0.34	0.22	1.00	1.47
JH	0.23	0.15	0.68	1.00

Table 4.5. Ghrelin fold change results from tissue culturing experiment.

	Fold change from each Treatment Group			
Tissue and Culture	DL	DH	JL	JH
DL	1.00	0.33	0.10	0.01
DH	3.07	1.00	0.32	0.03
JL	9.71	3.16	1.00	0.11
JH	92.41	30.06	9.51	1.00

Table 4.6. Ussing chamber results: means for flux, PD, G, ISC, and forskolin response (tissue viability) as compared using LSMEANS comparison.

Tissue	Culture	Flux (±SEM) (mM/cm^2/hr)	PD (±SEM) (mV)	G (±SEM) (millisiemen/cm^2)	ISC (±SEM) (μA/cm^2)	Forskolin (±SEM) (μA/cm^2)
Duodenum	None	117.07^a±31.62	0.57^a±0.14	19.03^a±2.85	10.03^a±2.06	10.82±1.23
Duodenum	High Glucose	277.68^b±36.51	0.39^a±0.14	29.28^{ab}±2.85	10.98^{ab}±2.06	6.37±1.23
Jejunum	None	136.47^a±36.51	0.34^a±0.14	18.30^{ac}±2.85	6.21^{abc}±2.06	-0.64±1.23
Jejunum	High Glucose	101.94^a±31.62	0.18^a±0.14	14.85^{acd}±2.85	2.23^{acd}±2.06	0.64±1.23

a-d values within columns with different letters differ (<0.05)

Table 4.7. mRNA expression results from tissues used in Ussing chamber experiment, compared using LSMEANS.

Tissue	Culture	mRNA Expression levels of Genes of Interest (±SEM)	
		GLUT2	SGLT1
Duodenum	No Culture	$-2.53^{a}\pm0.18$	$-3.89^{a}\pm0.08$
Duodenum	High glucose	$-1.51^{b}\pm0.18$	$-3.29^{b}\pm0.08$
Jejunum	No Culture	$-2.42^{ac}\pm0.18$	$-4.02^{ac}\pm0.08$
Jejunum	High glucose	$-1.13^{bd}\pm0.18$	$-2.80^{d}\pm0.08$

a-d values within columns with different letters differ ($p<0.05$)

Table 4.8. GLUT2 fold change results from tissues used in ussing chamber experiment (D0=duodenum no culture; DH=duodenum high glucose; J0= jejunum no culture; JH=jejunum high glucose).

	Fold change from each Treatment Group			
Tissue and Culture	D0	DH	J0	JH
D0	1.00	0.49	0.93	0.38
DH	2.03	1.00	1.88	0.77
J0	1.08	0.53	1.00	0.41
JH	2.64	1.30	2.45	1.00

Table 4.9. SGLT1 fold change results from tissues used in ussing chamber experiment.

	Fold change from each Treatment Group			
Tissue and Culture	D0	DH	J0	JH
D0	1.00	0.66	1.09	0.47
DH	1.52	1.00	1.66	0.71
J0	0.91	0.60	1.00	0.43
JH	2.13	1.40	2.33	1.00

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5.0 GLUT2, SGLT1, PEPT1 and Ghrelin mRNA Expression and Functional Glucose Transport in the Bovine Duodenum and Jejunum in Response to Varying Nutrient Concentrations in Relation to Animals with High and Low RFI Values

5.1 Introduction

How cattle distribute nutrients could have a major impact on their feeding behaviour. Kühn *et al.*, (2002) stated that once it can be established how different breeds distribute energy and nutrients in relation to their metabolizable energy, this will provide insight about nutrient pathways and their regulation. This statement, though general, could be thought of in relation to cattle with different residual feed intakes, and how nutrient transport and intake stimulation could affect those values. Based on the information attained from chapters 2 and 3, this chapter hopes to use tissue culturing, realtime PCR, and Ussing chambers to determine if differences exist between expression of the mRNA of the genes of interest (GLUT2, SGLT1, PEPT1, and ghrelin), glucose transport (measured by Ussing chambers), and steers that exhibit high and low residual feed intakes.

The objectives of these experiments are to determine whether steers that exhibit the extreme levels of RFI (positive and negative) differentially express mRNA as an indicator of the nutrient transporter genes: GLUT2, PEPT1, SGLT1, and the appetite stimulating peptide Ghrelin within duodenal and jejunal tissues cultured in a high nutrient bath. Cyclophilin was used as a reference gene to correct for the differing levels of RNA between samples. The null hypothesis for each of these genes is as follows: there is no difference in expression of mRNA representing the genes of interest (GLUT2, PEPT1, SGLT1, Ghrelin) within duodenal or jejunal tissues (cultured with their respective solutions i.e. high glucose for GLUT2, SGLT1, and Ghrelin and low glucose/high peptide for PEPT1) between steers with positive and negative RFI (α=0.05). The other objective of this experiment is to determine if there is a difference in the functionality of glucose transport using Ussing chambers. The null hypothesis for this objective is that there is no difference in the gene functionality of glucose in the duodenum or jejunum in steers with positive and negative residual feed intake.

5.2 Materials and Methods

5.2.1 Steers

Twelve steers used for this experiment were selected from a herd located at the University of Alberta Ranch, Kinsella, Alberta. The selection was based on RFI value and temperament. Temperament was a factor because the steers chosen needed to be halter broken and worked with in order to measure heat production at the University of Alberta Environmental and Metabolic Research Centre. Six of these steers had a negative RFI value while the other six had a positive RFI value. The steers and their RFI values are listed in table 5.1. The RFI values were based on the actual intake of each individual steer measured by the GrowSafe® system. The digestible energy of the feed was determined by a digestibility trial using sheep, where they were fed the same feed as the steers.

5.2.2 Tissue Samples

Duodenal and jejunal samples, approximately 1 meter in length, were collected from the steers at processing, approximately 10-15 minutes post slaughter. Once the tissue was removed it was placed in a plastic bag containing cold Krebs-Ringer bicarbonate buffer, this was done for both the duodenum and jejunum. The tissues were first ballooned with the Krebs solution (i.e. filled and sealed at either end). The bags of tissue were kept in a cooler full of ice. It is important to note that the samples remained intact in their cylindrical form (much like a sausage), with no incisions in the lining of the intestine, except for where it was removed. The tissue remained in this solution until preparation (~60-90 minutes).

5.2.3 Tissue Samples Preparation

Once the tissues arrived at the University campus laboratory they were first flushed with cold PBS to remove any digesta and viscous mucus left over in the intestine. They were then cut into two segments (approximately 20 cm in length), so they could used in their designated point of the experiment: high glucose (50 mM D-glucose), and high peptide/low glucose (20 mM L-carnosine/6 mM D-glucose). The high glucose and high peptide/low glucose tissue samples were clamped off at one side and then filled with their respective solutions (~120 ml). The solutions, were oxygenated and kept at 37°C before they were added to the tissues. Once the tissue samples were filled, they were clamped off at the other end so they looked like a sausage. These "sausages" filled with nutrient solutions were then placed in a water bath containing oxygenated Krebs-Ringer bicarbonate buffer and incubated at 37°C for twenty minutes. This time was determined by a time trial (section 7.10) in order to measure the optimum time of mRNA expression of GLUT2 and SGLT1 ($P<0.05$). After the time had passed the tissues were removed and cut open. The duodenal and jejunal tissues that were cultured in high peptide/low glucose were immediately scraped, snap frozen, and stored in separate tubes at -80°C. As for the duodenal and jejunal tissues that were cultured in the high glucose solution; a piece of each was cut off to be used for the Ussing chambers, the remainder of the tissue was scraped, snap frozen, and stored in separate tubes at -80°C. RNA was then extracted from these tissues and prepared for realtime PCR analysis in the same manner as described in chapter 2.

5.2.4 Ussing Chambers

Duodenal and jejunal tissue, after being cultured with the high glucose solution for 20 minutes, were mounted in Lucite chambers exposing mucosal and serosal surfaces to 10ml of oxygenated Krebs buffer maintained at 37°C by a heated water jacket and circulated by CO_2/O_2. Fructose (10mmol/L) was added to the serosal and mucosal sides. The spontaneous transepithelial potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing a short-circuit current (ISC) with an automatic voltage clamp (DVC 1000 World Precision Instruments), except for 5-10seconds every 5 minutes when PD was measured by removing the voltage clamp. Tissue ion conductance (G) was calculated from PD and ISC according to Ohm's Law. PD is expressed as millivolts (mV), Isc as microamperes per square centimeter ($\mu A/cm^2$), and G as millisiemens/cm^2. Baseline Isc and G were measured after a 20-minute equilibration period. Increases in Isc were induced by addition of the adenylate cyclase-activating agent, forskolin (105 μmol/L), to the mucosal surface. Epithelial responsiveness was defined as the maximal increase in ISC to occur within 5 minutes of exposure to the secretagogue.

In order to measure the basal flux 5 μCi [^{3}H] 3-O-methyl-D-glucose added to either the mucosal or serosal side after mounting and the tissue was allowed to equilibrate for 15 minutes; by this time the tissue was electrically stable. The reason the radioactively labeled glucose is added to either the mucosal or serosal side is because there are two pieces of each tissue mounted on the chambers so that a net flux can be determined, i.e. net transport activity. Bidirectional flux from mucosal-to-serosal and serosal-to-mucosal surfaces were determined for paired tissues by measuring four consecutive five-minute fluxes. The glucose flux is reported as the difference between the averaged values of the four consecutive five-minute fluxes. The net flux is the difference between the glucose flux measured from mucosal-to-serosal and serosal-to-mucosal.

5.2.5 Statistical Analysis

5.2.5.1 mRNA Expression Analysis

The analysis for the differences in mRNA expression was carried out using the PROC GLM procedure found in SAS (SAS Inst. Inc., 1996) and carried out by the following model:

$$Y_{jklmn} = \mu + C_i + R_k + CR_{jk} + D_l + W_m + \varepsilon_{jklmn}$$

Where Y_{ijkl} is the level of expression (ΔCt) of a mRNA of interest (GLUT2, SGLT1, PEPT1, or ghrelin) in a particular tissue (duodenum or jejunum), while μ is the overall mean of the tissue sample, and C_j is the fixed effect of the culture (high glucose; low glucose/high peptide). R_k represents the fixed effect of the residual feed intake group (steers with a negative RFI; steers with a positive RFI). CR_{jk} is the fixed effect of the interaction between culture and RFI group, D_l and W_m are covariates and represent the age of the dam and the day zero weight for each steer, and ε_{jklmn} is the residual error of the model. All F-tests were carried out using Type III sums of squares. Multiple comparisons of the level of expression of mRNA for each gene of interest for the interaction between culture and RFI group for each tissue were performed by the least squares method.

Finally, a correlation between the mRNA expression of the genes of interest, and between the mRNA expression of the genes of interest and the actual RFI values within each tissue was determined.

5.2.5.2 Ussing Chamber Analysis

The analysis for the differences in Ussing chamber results was carried out using the PROC GLM procedure found in SAS (SAS Inst. Inc., 1996) and carried out by the following model:

$$Y_{jkl,m} = \mu + R_j + D_k + W_l + \varepsilon_{jklm}$$

Where Y_{ijkl} is net flux of a particular tissue of interest, while μ is the overall mean of the tissue sample, Rj represents the fixed effect of the residual feed intake group (steers with a negative RFI; steers with a positive RFI), D_l and W_m are covariates and represent the age of the dam and the day zero weight for each steer, and ε_{iijk} is the residual error of the model. All F-tests were carried out using Type III sums of squares. Multiple comparisons of the level of net flux and RFI groups were performed by the least squares method.

5.3 Results

5.3.1 mRNA Expression

Messenger RNA expression analysis yielded no significant differences between the different treatment groups for any of the genes of interest located within the duodenum or the jejunum (tables 5.2 and 5.3 respectively). It is also important to note that the primer/probe set specific for ghrelin did not amplify properly for the treatments (both sets of tissues) of five steers. There were also no significant effects of culture or the covariates of dam age and day zero weight on any of the genes of interest.

In terms of correlations between the expression of the mRNA of the genes of interest, it was observed within the duodenum that GLUT2 was significantly positively correlated with SGLT1 (P=0.0035; r=0.57), but the correlation with PEPT1 only approached significance (P=0.0757; r=0.37). SGLT1 and PEPT1 on the other hand were strongly correlated (P<0.0001; 0.83). Ghrelin and RFI were not found to be correlated with each other, or any of the other genes of interest.

Correlations found within the jejunum were similar to those found within the duodenum. GLUT2 and SGLT1 were strongly correlated (P<0.0001; r=0.78) as were SGLT1 and PEPT1 (P=0.0004; r=0.66). In the jejunum, unlike the duodenum, the expressions of GLUT2 and PEPT1 were strongly correlated (P<0.0001; r=0.74). Ghrelin was significantly correlated with both GLUT2 and SGLT1 (P=0.0098; r=0.66; P=0.0074, r=0.68 respectively). RFI was not significantly correlated with any of the genes of interest.

5.3.2 Ussing Chambers

The ussing chamber results like the mRNA expression results showed no difference between the transport of glucose within duodenal or jejunal tissues. It is important to note that, because of time constraints (transport time) only 10 of the twelve animals could be measured in terms of transport capabilities. The covariates of dam age and day zero weight also had no significant effect on transport. The results from this experiment can be found in table 5.4.

5.4 Discussion

The results, in terms of variation of the mRNA expression of the particular genes of interest, do not appear to explain the differences in residual feed intake between different steers. There appears to be no statistically significant evidence that suggests the expression of GLUT2, SGLT1, PEPT1, and ghrelin within the duodenal and jejunal tissues differs between steers with positive and negative RFIs. It is also important to note that as in chapter 2 the standard error of GLUT2 was extremely high in both the duodenum and the jejunum, which leads to the conclusion that its level of expression is highly variable between different animals, but that the variation is not associated with RFI. The mRNA expression experiment revealed that the culturing technique appeared to have no effect on the expression of the genes of interest, although culturing was found to be significant in the time trial. In terms of the results attained from the Ussing chambers, it appears, from examining the means that overall the majority of the glucose passed through the tight junctions in the epithelium (hence the negative values), although there were some positive values found when comparing the individual animals. The standard error is also very high for these results, implying that there is a great deal of variability in glucose transport. The results of this experiment reveal that there appears to be no significant difference in glucose transport between animals with positive and negative residual feed intakes.

The correlations between the mRNA expression levels of the genes of interest from these experiments appear to be similar to those found in chapter two. The positive correlations between GLUT2, SGLT1, and PEPT1 (almost significant correlation between GLUT2 and PEPT1 in the duodenum) reveal that steers with a high mRNA expression of any of the transporter genes of interest will have a high expression of the any of the other transporter genes of interest. Ghrelin, although not significantly correlated with anything in the duodenum was found to be positively correlated with the glucose transporter genes much in agreement with the positive correlation with GLUT2 observed in chapter 2. Although there is nothing published in the literature on the relationship between these genes, it would appear that animals that have high expression of mRNA of GLUT2 or SGLT1 would have high expression of ghrelin mRNA as well.

Reasons for there not being any differences in mRNA expression of the transporter genes, ghrelin, and actual glucose transport between animals with differing residual feed intakes could be due to time lag. The time it took to get the tissues and bring them back to the lab for analysis took about 60-90 minutes. Added to this the tissue had to be incubated for twenty minutes. The time frame in which the tissue is sitting in the buffer solution needs to be decreased. Tissue can be kept alive in this situation, but it is slowly dying. If there was some way of getting the tissue from the animal and culturing it on route to the lab, this could greatly increase the chances of observing upregulation of the particular genes of interest (GLUT2, SGLT1, PEPT1, and ghrelin). The study by Cui *et al.* (2003) perfused glucose before euthanizing rats, and found upregulation of GLUT2 expression. This is somewhat difficult to do when dealing with cattle; however cannulation of the animal may be a solution when dealing with such large animals. Biopsies of the intestinal wall could be taken through a cannula after nutrients were infused into the small intestine.

The fact that ghrelin did not amplify in the tissues of 5 of the steers being studied could lead to one of the following conclusions: the animals were not hungry when they were slaughtered; stress caused a lack of appetite thus directly affecting ghrelin; the mRNA was degraded; or there is a polymorphism in the ghrelin sequence. There is a possibility that the animals were not hungry when they arrived at the slaughter facility, but there still would have been an expression of mRNA as an indicator of the gene even if it was low. Stress appears to increase the expression of ghrelin. This was demonstrated by (Asakawa *et al.*, 2001), who showed that causing an outward stress on a mouse (tail pinch) increased the expression of ghrelin in the stomach. The mRNA could not be degraded because the cyclophilin and the mRNA of the other genes amplified. The most likely of these options is that there is a polymorphism in the sequence of ghrelin in either the primer or probe sight. The results attained from the non amplified product are similar those of earlier experiments where SGLT1 would not amplify. In this case there was one base difference in the forward primer and this caused little to no amplification. The fact that there may be a polymorphism in ghrelin is promising; this could lead to many more studies. This polymorphism has the possibility of relating to many different factors in cattle production, much like the leptin polymorphism (Nkrumah *et al.*, 2003). Polymorphisms in ghrelin appear to have links to obesity in people (Hinney *et al.*, 2002; Korbonits *et al.*, 2002; Ukkola *et al.*, 2002). This could be very interesting in terms of cattle; these polymorphisms could have relations to residual feed intake.

The messenger RNA expression as an indicator of the genes GLUT2, SGLT1, PEPT1, and ghrelin do not appear from these results to show any significant differences within the duodenum and jejunal tissue of steers with positive and negative residual feed intakes. The transport of glucose also appears to not be significantly effected by RFI based on the Ussing chamber methods used in this experiment. Some suggestions in the final discussion and conclusion look at other aspects that could be considered when looking at these particular genes of interest.

Table 5.1. ID Numbers, residual feed intakes (kg), age of dam (year), day zero weight (kg) of the steers used in for this experiment.

Animal	RFI	DAMAGE	DAY0WT
19	0.20	7	313.49
25	-1.90	9	283.78
39	-1.74	9	290.05
61	-1.58	9	303.80
63	-2.04	7	351.24
131	-0.23	7	339.70
149	1.59	7	335.05
175	1.72	9	326.08
185	0.77	8	301.19
195	1.04	9	316.83
393	0.87	6	355.38
499	-0.69	6	371.31

Table 5.2. mRNA expression results within the duodenum; mean values and comparison using LSMEANS.

		Δ Ct (±SEM)			
RFI Group	Culture	GLUT2	SGLT1	PEPT1	Ghrelin
Negative	LG/HP	2.91 ± 2.19^a	-2.21 ± 0.75^a	-2.28 ± 0.48^a	1.83 ± 0.22^a
Negative	HG	3.01 ± 2.19^a	-2.53 ± 0.75^a	-2.13 ± 0.48^a	2.09 ± 0.22^a
Positive	LG/HP	0.77 ± 2.19^a	-1.64 ± 0.75^a	-1.81 ± 0.48^a	1.92 ± 0.19^a
Positive	HG	1.13 ± 2.19^a	-1.87 ± 0.75^a	-1.72 ± 0.48^a	1.98 ± 0.19^a

a-d Values within the same column with different letters differ ($P<0.05$)

Table 5.3. mRNA expression results within the duodenum; mean values and comparison using LSMEANS.

		Δ Ct (±SEM)			
RFI Group	Culture	GLUT2	SGLT1	PEPT1	Ghrelin
Negative	LG/HP	1.48 ± 1.68^a	-1.82 ± 0.73^a	-2.55 ± 0.60^a	4.49 ± 1.03^a
Negative	HG	0.70 ± 1.68^a	-1.92 ± 0.73^a	-2.95 ± 0.60^a	5.00 ± 1.03^a
Positive	LG/HP	0.05 ± 1.68^a	-1.92 ± 0.73^a	-2.84 ± 0.60^a	5.15 ± 0.90^a
Positive	HG	-0.17 ± 1.68^a	-2.10 ± 0.73^a	-3.05 ± 0.60^a	5.78 ± 0.90^a

a-d Values within the same column with different letters differ ($P<0.05$)

Table 5.4. Net flux results ($mM/cm^2/hr$) within duodenal and jejunal tissues; mean values and comparison using LSMEANS.

RFI Group	Tissue	Net FLUX (SEM)
Negative	Duodenum	-18.93±39.97
Positive	Duodenum	-70.99±42.96
Negative	Jejunum	-42.68±49.66
Positive	Jejunum	-38.67±44.23

a-b Values within the same tissue with different letters differ ($P<0.05$)

5.5 References

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6.0 General Discussion and Conclusions

6.1 General Discussion

The ability to process nutrients varies between cattle (Archer *et al.*, 1998; Arthur *et al.*, 1998; Okine *et al.*, 2001). The original goal of this thesis was simple: to determine if the overall expressions of mRNA as an indicator of the genes of interest (GLUT2, SGLT1, PEPT1, and ghrelin) were expressed differently and that glucose was transported differently within duodenal and jejunal tissue of steers at the extreme ends of residual feed intakes. This turned out to be a task much more difficult than at first conceived. Although the overall goal was not achieved, some interesting facts became apparent through these studies.

There appears to be significant positive correlations between the expression of mRNA of the transport genes, especially between GLUT2 and the other genes. These correlations would imply that when a steer has a higher overall expression of GLUT2 within the duodenum it will have a higher overall expression of SGLT1 (Chapter 2, r=0.44; Chapter 4, r=0.57), and PEPT1 (chapter 4, r=0.37 approaching significance) within the duodenum, and there appears to be a strong correlation between the overall expression of SGLT1 and PEPT1 (chapter 4, r=0.83) within the duodenal tissue of the steers. The relationship between ghrelin and GLUT2 within the duodenum did not appear to be significant in chapter 4, however it was found to be significant in chapter 2 (r=0.43). Strong positive correlations were also found in the jejunum between GLUT2 and both SGLT1 and PEPT1 (r=0.78 and 0.74 respectively) as well as between SGLT1 and PEPT1 (r=0.66). In the jejunum ghrelin had a significant positive correlation with both GLUT2 and SGLT1 (r=0.66 and r=0.68 respectively). The implications of the correlations found between the expression of mRNA of the respective transport genes would lead one to believe that when an animal expresses one of them highly, it will express the other ones as well. This could lead to an assumption that the messenger RNA of these genes would all be expressed at higher levels while nutrients are passing through the respective segments. The correlations found between ghrelin and the transport genes SGLT1 and GLUT2 within the jejunal samples cannot not be considered as accurate as the others, because not all of the animals were used to determine those correlations.

The effect of culture although not having a significant effect on the expression of the mRNA of the genes of interest between animals with differing residual feed intake values, highly effected the expression of ghrelin within the duodenal and jejunal tissue of a dairy cow. The tissue was cultured longer in that experiment and this may be an indication as to what needs to be done in the future when studying ghrelin. Ghrelin is affected by intake (Tschöp *et al.*, 2000). The expression of ghrelin throughout the bovine gastrointestinal tract is highest within the abomasum, and second highest within the duodenum (chapter 3).

Although this study did not find any differences between the expressions of the mRNA as an indicator of the genes of interest within the duodenum and jejunum of steers with high and low residual feed intakes, this area of study should not be discarded. The statement by Kellems and Church (2002), that it is more efficient for cattle to retain energy from carbohydrates digested and absorbed directly in the small intestine than retain the energy that is produced by VFAs, leads one to believe that the small intestine is still a major area of interest, when considering animals of differing residual feed intakes. The suggestions for further study touches on methods that should be considered and hopefully improve this area of research and aid in the discovery of some type of marker that could be selected for when looking for animals with negative residual feed intakes.

6.2 Conclusions

Upon review the data from this thesis some tentative conclusion can be reached. First of all I found no significant relationship between residual feed intake and the overall expression of mRNA indicative of the transport genes GLUT2, SGLT1, and PEPT1, as well as the intake stimulating peptide ghrelin within frozen duodenal samples of steers with high and low residual feed intakes. When comparing tissues of a dairy cow there were differences found in mRNA expression of the transport genes as well as ghrelin which lead to speculation that culturing duodenal and jejunal tissue in a high glucose or low glucose/high peptide solution does have a significant effect on their expression. However, I found that culturing does not have a significant effect on the overall expression of the mRNA of in terms of the genes interest when comparing their expression within the duodenum and jejunum of steers with differing residual feed intakes. I still believe that this is an area of study worth looking into for further research, and the next section deals with suggestions that should be considered for further study.

6.3 Suggestions for further study

During the course of this thesis, a number of interesting aspects for improving further research became apparent in the hopes that they will aid in the discovery of reasons for cattle with differing residual feed intakes. These include:

1. *In Vivo* study – Originally part of this thesis was going to involve a cannulation study of the effects of infusing a nutrient rich solution (high in glucose, and peptides) into the abomasum and biopsies of regions of the small intestine examined for differences in both total protein and gene expression. This would provide one of the first *in vivo* study of the expression of transporter mRNA within the digestive tract of a cow. Not only would the gene expression results be helpful in determining differences, but the total protein expression results would be able to explain things in which the gene expression results might have fallen short.
2. Microarray or Oligonucleotide Arrays – The microarray and the oligonucleotide array allow for the scanning of a larger number of genes (Schena *et al.*, 1996; Lipschutz *et al.*, 1999). Each gene (probe) is hybrodized to a glass slide or a chip, and then fluorescent targets (cDNA labeled targets representative of mRNA from specific tissue of a specific animal) are allowed to hybridize to these probes. The differences in fluorescence represent the different levels of expression. Arrays of the small intestine could be fabricated in order to test the effects of thousands of genes and narrow down what needs to be investigated in greater detail, and what can be ruled out.
3. Protein Analysis – There are limitations to solely studying the expression of mRNA in terms of the turnover rate. The amount of mRNA present in a cell at a particular point in time is dependent on two factors: the rate at which it is being produced and the rate at which it is being used up (translated into protein). This could lead to problems in solely examining the amount of mRNA because it may not be indicative of the amount of the actual protein it is coding for. A study performed by Lescale-

Matys *et al.* (1993) found that although the expression of SGLT1 mRNA within the lamb intestine when 30 mM of glucose was infused into the small intestine only increased 2 fold, the actual protein expression of that particular transporter increased 60-90 fold. This was also pointed out by Cho-Leam (1994), and leads one to believe that although the mRNA is important to examine in terms of expression, protein level would also be a key area of interest. There are two major types of analysis methods that I would suggest for this type of study: western blot or 2D gel electrophoresis. A western blot can determine the amount of a specific protein present in a particular tissue, and if there is an antibody specific to a particular protein then this type of analysis would be ideal. However, if there is not a specific antibody for the protein of interest or if the researcher would like to perform a total protein scan for the particular area of interest a 2D gel can be used. It separates the proteins by charge and then by size so that a large number of proteins can be scanned at once. Whichever methods are used, it is important that protein level be considered when examining the expression of mRNA on a large scale (i.e. genome scan), or a small scale (i.e. individual genes).

4. Further Investigation into Ghrelin – The study of ghrelin since its discovery has raised many interesting questions. Ghrelin not only controls feed intake, but it also has effects on growth hormone. In all of the studies reviewed only one dealt with ghrelin in relation to cattle. Hayashida *et al.* (2001) used an RIA to determine the levels of plasma ghrelin before and after feed in seven adult black Japanese cows. This RIA could be useful in determining differences between the cattle whose residual feed intakes are known. It could be possible that animals with differing

residual feed intakes expression ghrelin levels in plasma differently. Ghrelin's failure to amplify in the tissues belonging to five animals in chapter 4 would lead one to speculate that a polymorphism exists within the gene. This could prove to be interesting if different cattle exhibit these polymorphisms in terms of growth traits, intakes, or meat quality characteristics.

5. Ileum – The ileum could be an area of interest in terms of nutrient transport between animals with differing residual feed intakes. Although it is thought as a compensatory tissue, which absorbs the left over glucose that was not absorbed up by the duodenum and jejunum (Ferraris and Diamond 1986), this may play an important role in residual feed intake. It is possible that efficient animals with negative residual feed intake have ileums that are more active in recovering the left over nutrients than those with positive residual feed intakes. There may be a higher expression of GLUT2, SGLT1, or differences in transport capabilities.
6. Aperture Size – Upon consulting with Dr. Karen Madsen on possible ways to improve the experiment in the future she brought up the issue of aperture size. Currently the aperture size used in the Ussing chambers is 5 mm, by 15mm. This size works fine for rats, mice, and pigs, but there are larger sizes available and they might be better for cattle.

6.4 References

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7. Appendices

7.1 RNA Preparation

The TRIZOL (*Invitrogen*) technique was used to isolate RNA from the individual tissue samples. Each sample was taken from the freezer and ground up using a mortar and pestle set in dry ice. Liquid nitrogen was added in order to keep the sample from thawing. About 500 mg was taken from the ground sample and the rest was stored and returned to the –80°C freezer. The 500 mg was placed into a Falcon tube with 5 ml of TRIZOL (1 ml TRIZOL/100 mg of tissue) and left to stand for 5 minutes at room temperature. One ml of chloroform (0.2 ml chloroform/1 ml TRIZOL) was added and the tube was shaken vigorously for 15 seconds and then left to stand for 2-3 minutes at room temperature. The tube was then placed in a centrifuge and spun for 20 minutes at 10000 RPM. The centrifugation separated the solution into 3 phases (RNA, DNA, and protein). The phase containing the RNA, i.e. the clear supernatant, was removed and placed in a new Falcon tube, while the remainder of the solution was discarded. Two and a half ml of propanol (0.5 ml propanol/1 ml TRIZOL) was added to the clear RNA solution in order to pellet the RNA. This was left to sit at room temperature for 10 minutes. The tube was then spun at 10000 RPM for 15 minutes resulting in the formation of a pellet at the bottom of the tube. The supernatant was removed and the pellet was washed with 5 ml of ethanol (1 ml ethanol/1 ml TRIZOL) and spun at 7500 RPM. The pellet was then air dried and re- dissolved in 400µl of water. An OD measurement was then taken on the RNA solution at a 1:200 dilution in order to determine the concentration. Finally, the RNA was divided into two tubes, one of which contained sodium acetate and 100% ethanol, and stored at –80°C. The other aliquot was adjusted to a concentration of 100 ng/ul and stored at –20°C to be used for this experiment.

7.2 DNase Treatment

In order to minimize errors resulting from the presence of genomic DNA, the RNA samples were treated with DNase. One µl of DNase I was combined with 1 µl of 10x DNase I reaction buffer and 10 µl of RNA. This mixture was then left to stand for 15 minutes, following which 1 µl of stop solution (25 mM EDTA) was added. After the addition of the EDTA, the solution was incubated at 65°C for 10 minutes and then placed on ice.

7.3 Reverse Transcription

Once the RNA has been treated to remove genomic DNA it is ready to be reverse transcribed. The reverse transcription involved adding 2 µl of 10x PCR reaction buffer, 2 µl of $MgCl_2$, 1 µl of 20 mM dNTPs, 0.5 µl of 1 µg/µl of oligo dT, 0.5 µl of an RNase inhibiting enzyme (RNase out; supplied by *Invitrogen*), and 1µl of a reverse transcriptase (Superscript II; supplied by *Invitrogen*) to a total volume of 20 µl. The mixture was then placed in a thermalcycler where it was held at 25°C for 10 minutes. Next it was heated to and held at 48°C for 30 minutes, and finally the temperature was increased to 95°C and held for 5 minutes. In order to stop the reaction after the final hold at 95°C, the tube containing the solution was placed on ice. Finally the concentration of the solution was adjusted to 5 ng of RT per µl by adding 180 µl of water. The samples were then stored in the -20°C freezer until needed for the realtime PCR experiment.

7.4 MGB Primer and Probe Design

The real-time PCR primers and minor groove binding (MGB) probes used for the bovine genes of interest and bovine cyclophilin, a housekeeping gene used as a control (Miller *et al.*, 2000; Lindqvist *et al.*, 2003), were designed using the *Primer Express™ 1.5* software package (*Applied Biosystems Inc.*). It is important to note that the partial sequence for bovine PEPT1 was found by running a blast search through the TIGR data base using the human sequence; accession number XM_007063 (Gish, 1996-2000). The probes were labeled with different fluorophores for multiplex amplification. The genes of interest were labeled with a FAM-fluorophore while cyclophilin was labeled with a VIC-fluorophore. As part of the probe design process, the sequences of the primer-probe sets corresponding to the genes of interest and cyclophilin were compared to each other using the *Amplify 1.2* software (Engels, 1993). This was done to assure that the primers/probe for one gene did not bind to the sequence of the other gene. The sequences for the primers/probe set for each of the genes of interest can be found in Table 7.1, and a representation of each sequence and where the primer/probe set binds to its respective sequence is found in figures 7.1 through 7.5 (7.1 GLUT2; 7.2 SGLT1; 7.3 PEPT1; 7.4 Ghrelin; 7.5 Cyclophilin).

In order to test if the primer/probe sets were specific to the sequences of the genes of interest, they were tested individually at first, before being multiplexed with cyclophilin. The optimum primer/probe concentrations were determined by use of a concentration grid where different concentrations of primers and probe, in combination with cDNA generated by the reverse transcription of mRNA, were compared on the realtime PCR. After the optimum concentration conditions were determined, the primer/probe sets for the genes of interest were then run in a multiplex reaction with cyclophilin to determine if there were any losses in amplification in comparison to that which was attained from only amplifying one primer/probe set per reaction. Upon analyzing the results it was determined that multiplexing the genes of interest and cyclophilin had little to no effect on the amplification of the other's PCR product.

Table 7.1. Dye label, forward and reverse primer and probe for each of the genes of interest.

GENE	DYE	Primer-1(Forward) 5'-3'	Primer-2(Reverse) 5'-3'	Probe 5'-3'
GLUT2	FAM	GCTCTTCACCAATGCCAGCTA	CCGCATGCAGCATCAATG	CGACAGCCTATTCTAG
SGLT1	FAM	ACTGCGAGGCCTGATGCT	ATGGAGGTCAGCGAGCTCAT	ATGCTGGCCTCGCT
PEPT1	FAM	GGAGCAGGCCAGTTCAGTGA	GCAACGCCGCAAACAGA	AGTGGGCCGAGTACG
GHRELIN	FAM	CGAGCTGGAAATCCGGTTC	CCCTGCTAGCTTGATCCCAA	CGCCCCCTTTAAC
CYC	VIC	CCCAGGCTAAGCCTTCCAA	CCGATGTCCACGTCGAAGA	CCCAGTAACCCTCGAGTT

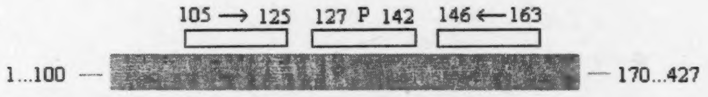

Fig. 7.1. GLUT2 primer/probe set location on partial bovine GLUT2 reverse transcribed mRNA sequence (accession number AF308828).
(→ represents the forward primer, P represents the probe, and ← represents the reverse primer)

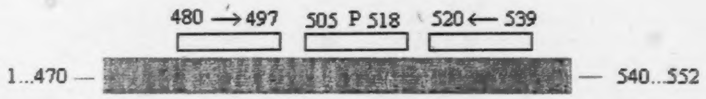

Fig. 7.2. SGLT1 primer/probe set location on partial bovine SGLT1 reverse transcribed mRNA sequence (accession number AF308831).
(→ represents the forward primer, P represents the probe, and ← represents the reverse primer)

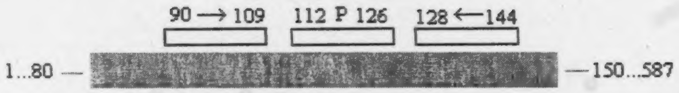

Fig. 7.3. PEPT1 primer/probe set location on partial bovine PEPT1 reverse transcribed mRNA sequence (accession number BF044840).
(→ represents the forward primer, P represents the probe, and ← represents the reverse primer)

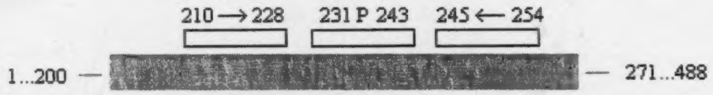

Fig. 7.4. Ghrelin primer/probe set location on partial bovine ghrelin reverse transcribed mRNA sequence (accession number AF350329).
(→ represents the forward primer, P represents the probe, and ← represents the reverse primer)

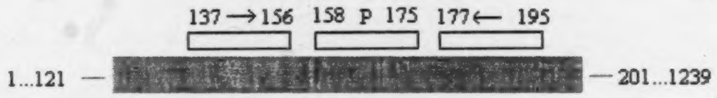

Fig. 7.5. Cyclphilin primer/probe set location on complete bovine cyclophilin reverse transcribed sequence (accession number D14074).

7.5 Realtime PCR

The amplification of the gene of interest and cyclophilin specific product was carried out with an *ABI PRISM™ 7700 Sequence Detector* (*Applied Biosystems Inc.*). Each real-time PCR reaction contained 12.5 µl of a universal master mix supplied by *Applied Biosystems Inc.*, 3.5 µl of DEPC-treated water, 0.225 µl of the forward and reverse primers for cyclophilin (100 µM), 0.05 µl of the VIC-labeled fluorescent cyclophilin probe (100 µM), 1.5 µl of the forward and reverse primers for the gene of interest (10 µM), 0.5 µl of the FAM-labeled fluorescent probe for the gene of interest, and 5 µl of the rt solution (i.e. 50 ng of original total RNA) for a total volume of 25 µl per reaction. Three replicates were used for each gene of interest per tissue per animal. The average of the three replicates is used for data analysis. The conditions for the real-time PCR are listed in table 7.2.

Table 7.2. Real time PCR conditions.

Time	Temperature	Stage	Setting
2 min	50°C	Initial	Hold
10 min	95°C	Initial	Hold
15 sec	95°C	Denature	Cycle (40x)*
1 min	60°C	Anneal/extend	Cycle (40x)*

*The temperatures are cycled between each other

7.6 Data Correction

The threshold cycle or Ct value is the cycle at which a statistically significant increase in ΔRn is first detected. ΔRn indicates the magnitude of the signal generated by the given set of PCR conditions. The Ct value occurs when the sequence detection system begins to detect the increase in signal associated with an exponential growth of PCR product. This means that, in order to accurately measure amplification, the threshold level is set where the signal first begins to become linear. The data is corrected according to *User Bulletin #2* of the *ABI PRISM™ 7700 Sequence Detection System* (*Applied Biosystems Inc.*, 1997). The Ct value of cyclophilin for each treatment is subtracted from the Ct value for each gene for each treatment, known as the ΔCt values. It is important to note that the lower the ΔCt value the higher the expression of that particular gene of interest.

7.7 Fold Change Calculation

The $2^{-\Delta\Delta Ct}$ method is used to determine fold change if the differences in ΔCt are significantly different, as per *User Bulletin #2* of the *ABI PRISM™ 7700 Sequence Detection System* (*Applied Biosystems Inc.*, 1997). This system utilizes the data corrected values determined by the subtraction of cyclophilin. The average ΔCt of the gene within the treatment group that has the lowest expression level of mRNA of the gene is then subtracted from the average ΔCt of each of the remaining treatment groups for that particular gene. This is referred to as the ΔΔCt value. The ΔΔCt value is then used in the formula $2^{-\Delta\Delta Ct}$ to determine the fold change.

7.8 Krebs Ringers Solution

Table 7.3. Final Concentrations for reagents used to make Krebs-Ringer Bicarbonate Buffer

Reagent	Final Concentration (mM)
NaCl	110.0
CaCl2	3.0
KCl	5.5
KH2PO4	1.4
NaHCO3	29.0
Na-Pyruvate	5.7
Na-Fumarate	7.0
Na-Glutamate	5.7
Mannitol	1.0
*Dextrose	50.0
**L-Carnosine	20.0
**Dextrose	6.0

* Added to make high glucose solution
**Added to make low glucose/high peptide solution

7.9 Calculating Residual Feed Intake

7.9.1 GrowSafe® System

The GrowSafe® system (GrowSafe Systems Ltd., Airdrie, Alberta) enables the measuring of daily feed intake for each individual steer. Each steer is fitted on his ear with a transponder that carries its electronic identification (EID). There are 10 feed bunks each of which are on scales that measure the weight of feed in the bunk constantly. When an animal decides to eat it is detected at the feed bunk and the GrowSafe® system measures how much the animal eats. A more detailed description of the GrowSafe® system can be found in Basarab *et al.* (2000). This feeding trial endured for 71 days in order to determine the average daily intake for each steer.

7.9.2 Digestible Energy of Feed

The digestible energy (DE) of the feed was determined by conducting a digestibility trial consisting of three sheep and the feed that is fed to the steers out at the Kinsella ranch. The sheep were placed in individual crates so that their feed intake and faecal output could be measured. This was done for two five day periods. Each day the faeces of each sheep was collected and weighed, as well as the feed that it left behind (orts). After each was weighed a sample was taken and stored in a freezer. There was one container for each sample (faecal and orts) per animal per 5 day period. Samples of the feed were also taken throughout the trial for each 5 day period. Once both feed trials were finished the dry matter of each sample was determined both at 65°C and 100°C respectively. The samples were ground before they were dried at 100°C. The dry matter of the feed was determined to be 93.5%, which means that 6.5% of the weight of the feed can be attributed to moisture. Once the dry matter of the faecal, feed, and orts samples for each animal for each period was established, the amount of energy of each sample was determined using bomb calorimetry. In order to calculate the digestible energy of the feed the dry matter was multiplied by the average daily feed intake (kg/day) and then converted into multiplied by the energy level of the feed (Kcal/g) in order to determine the amount of energy intake per day. It is important to note that the values used were the average for each individual sheep for both feeding trials. The faecal energy expenditure per day (Kcal) was determined in a similar matter, the energy of the faecal matter was multiplied by the dry matter weight of the faeces. The digestible energy was determined by subtracting the energy of the faecal matter from the energy of the feed and then multiplied by 4.184 to convert from kilocalories to kilojoules, and then divided by one thousands to convert to megajoules (MJ). The DE was then divided by the amount of kilograms of the feed consumed to determine the DE in megajoules/kg. This was done for each individual sheep. An average was then taken and the final DE of the feed was determined to be 14.77 MJ/kg.

7.9.3 RFI Calculation

Residual feed intake was calculated by the method described in section 2.1.3. In order to determine observed feed intake, some initial values are needed. Total intake was calculated by multiplying the average intake by 71 (i.e. total number of days on the trial). This value is then multiplied by 0.935 (%DM), and then converted to total metabolizable energy intake (MEI). This was done by multiplying the total DMI by the metabolizable energy of the diet, 12.12 MJ/kg (82% of the digestible energy calculated from bomb calorimetry). The MEI was then standardized by adjusting each animal's MEI into standard feed intake at 10 MJ/kg. Finally the observed feed intake was then converted into daily standard feed intake by dividing by the days on trial.

Expected feed intake (EFI) was then determined by calculating the average daily gain (ADG) and the metabolic midweight (MMIDWT) for each animal, following which they are regressed on observed feed intake. After OFI and EFI have been calculated the RFI for each animal was be determined.

7.10 Time Trial

A time trial was conducted in order to determine the optimum culture time at which an upregulation in the expression of glucose transporter (GLUT2 and SGLT1) mRNA in duodenal and jejunal tissue occurs, when cultured with a high glucose solution, compared to tissue cultured with a low glucose concentration. It is important to note that the sole purpose of this study was to determine the time point when a increase in expression occurs and thus only tissues within each time point are relevant to this comparison.

Duodenal and jejunal tissue samples were taken from two beef steers at slaughter. These tissues were placed in a bag filled with a Krebs-Ringers solution so that they could be transported back to the University of Alberta. The tissues were cut into sections and then cultured with either a high glucose solution or a low glucose/high peptide solution. The tissues were then incubated in a water bath filled with Krebs-Ringers solution at 37°C. Each tissue was cultured (in duplicate; i.e. one from each animal) for specific times (10, 20, and 30 minutes).

The results from this experiment can be found in Table 6.5. These results show that although upregulations in expression of mRNA occurred within other treatment groups, the jejunal tissue at the twenty minute time point was found to be the highest for both GLUT2 and SGLT1. The jejunal tissue cultured with the high glucose solution expressed GLUT2 and SGLT1 1.84 and 1.75 times higher than the jejunal tissue treated with the low glucose/high peptide solution.

Based on these results it was determined that the tissues should be cultured for twenty minutes with the respective solutions in the hopes that differences will be detected between steers with high and low residual feed intakes.

Table 7.4. Time trial results for the expression of GLUT2 and SGLT1 as compared using LSMEANS.

				ΔCt (±SEM)	
Comparison	Tissue	Culture	Time	GLUT2	SGLT1
1	Duodenum	L	10	-3.80 ± 0.18^{a}	-4.75 ± 0.23^{c}
1	Duodenum	H	10	-3.74 ± 0.18^{a}	-5.11 ± 0.23^{d}
2	Duodenum	L	20	-3.45 ± 0.18^{a}	-4.04 ± 0.23^{c}
2	Duodenum	H	20	-3.53 ± 0.18^{a}	-4.22 ± 0.23^{c}
3	Duodenum	L	30	-3.31 ± 0.18^{a}	-3.52 ± 0.23^{c}
3	Duodenum	H	30	-3.27 ± 0.18^{a}	-3.55 ± 0.23^{c}
4	Jejunum	L	10	-2.95 ± 0.26^{a}	-3.14 ± 0.23^{c}
4	Jejunum	H	10	-3.39 ± 0.18^{b}	-3.53 ± 0.23^{b}
5	Jejunum	L	20	-3.25 ± 0.18^{a}	-3.34 ± 0.23^{c}
5	Jejunum	H	20	-4.13 ± 0.18^{b}	-4.14 ± 0.23^{d}
6	Jejunum	L	30	-2.86 ± 0.18^{a}	-2.73 ± 0.23^{c}
6	Jejunum	H	30	-3.22 ± 0.18^{b}	-2.98 ± 0.23^{c}

a-b GLUT2 Means of the same comparison group with different letters differ ($p<0.05$)
c-d SGLT1 Means of the same comparison group with different letters differ ($p<0.05$)

7.11 References

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